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Manuscript Details

Manuscript number	CCR_2016_292
Title	Mass spectrometry as a powerful tool to study therapeutic metallodrugs speciation mechanisms: current frontiers and perspectives
Article type	Review Article

Abstract

Metal-based compounds form a promising class of therapeutic agents, whose mechanisms of action still need to be elucidated, and that are in general prone to undergo extensive speciation in physiological environment. Thus, determination of the fate of the metal compounds in complex biological systems, contributing to their overall pharmacological and toxicological profiles, is important to develop more rationalised and targeted metal-based drugs. To these aims, a number of spectroscopic and biophysical methods, as well as analytical techniques, are nowadays extensively applied to study the reactivity of metal complexes with different biomolecules (e.g. nucleic acids, proteins, buffer components). Among the various techniques, molecular mass spectrometry (MS) has emerged in the last decade as a major tool to characterise the interactions of metallodrugs at a molecular level. In this review, we present an overview of the information available on the reactivity of various families of therapeutic metallodrugs (mainly anticancer compounds based on Pt, Ru, Au and As) with biomolecules studied by different MS techniques, including high-resolution ESI-, MALDI- and ion mobility-MS among others. Representative examples on the potential of the MS approach to study non-covalent interactions are also discussed. The review is organized to present results obtained on samples with different degrees of complexity, from the interactions of metal compounds with small model nucleophiles (amino acids and nucleobases), model peptides/oligonucleotides, target proteins/nucleic acids, to the analysis of serum, cell extracts and tissue samples. The latter requiring combination of proteomic methods with advanced MS techniques. Correlations between molecular reactivity of metallodrugs and biological activity are hard to establish, but differences in the reactivity of metallodrugs to biomolecules and their different adducts, as revealed by MS methods, may indicate differences in their modes of action. Overall, the knowledge offered by MS methods on metallodrugs speciation is invaluable to establish new rules and define new trends in the periodic table aimed at rationalizing the behavior of metal compounds in complex living systems.

Keywords	metal-based drugs; proteins; nucleic acids; mass spectrometry; metallomics.

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Answers to Reviewers_24-12-2016.docx [Response to reviewers]

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Answers to Reviewers

Reviewer 1

The authors have made some effort to answer suggestions and the manuscript is improved. Since it is a review, there is, strictly, no new information but nevertheless the compilation of data and references could be useful.

Answer: no further comments.

Reviewer 2

This topical review provides a complete overview of metallodrug interaction with biomolecules, with particular emphasis on speciation analysis by mass spectrometry, which represents a valuable tool for the molecular characterization of adducts and the elucidation of binding sites of metallodrugs in proteins and nucleic acids.
The review is well organized with a good integration between different sections, going from model biomolecular systems to complex samples (cells and tissues). The scientific context for each class of metal compounds is well outlined, and the results of analyses are clearly described.
A good balance of cited literature allow to critically assess the importance of the achieved results.
After a brief reference to the technical description of methods, the review encompasses the most recent advancements in the field. The authors make clear that each technique has to be used in combination with other approaches to avoid biased conclusions.
Next to inevitable limitations and artefacts, especially in the case of non-covalent and outer sphere interactions between protein targets and metal compounds, mass spectrometry shows a high potential in the analysis of complex mixtures, which still needs to be fully exploited.
Minor points:

i) Page 4: As far as I know, clinical trials on NAMI-A were terminated
ii) Page 41: Cu(0) is not a toxic ion. Did the authors mean copper nanoparticles or Cu(II) ions?
iii) Page 42: I noticed some typo errors.
Use "Copper transport proteins" in place of "Copper transporters proteins"

"chemotherapeutics" in place of "chemotherapeuties"

"two Cu(I) exporting ATPases"

"hCtr1 peptide chains"

[peptide+Pt(OH2)]2+

"Liu et al." in place of "Lu et al."
iv) Some symbols in the PDF file are not correctly displayed.

Answer: We have inserted all the minor modifications requested by this reviewer. Furthermore, we have carefully revised once more the entire manuscript to correct typos, avoid repetitive sentences, polish the English and correct symbols.

-Reviewer 3

- This is a much improved review that should be accepted once the authors have provided a list of abbreviation to be added right after the abstract and keywords and the issues with symbols mentioned by reviewer 2 has been corrected.

Answer: As requested by this reviewer and by the Editor, we have added a list of Abbreviations before the introduction.

“Mass spectrometry as a powerful tool to study therapeutic metallodrugs speciation mechanisms: current frontiers and perspectives“

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Outline

Abstract

Metal-based compounds form a promising class of therapeutic agents, whose mechanisms of action still need to be elucidated, and that are in general prone to undergo extensive *speciation* in physiological environment. Thus, determination of the fate of the metal compounds in complex biological systems, contributing to their overall pharmacological and toxicological profiles, is important to develop more rationalised and targeted metal-based drugs. To these aims, a number of spectroscopic and biophysical methods, as well as analytical techniques, are nowadays extensively applied to study the reactivity of metal complexes with different biomolecules (e.g. nucleic acids, proteins, buffer components). Among the various techniques, molecular mass spectrometry (MS) has emerged in the last decade as a major tool to characterise the interactions of metallodrugs at a molecular level.

In this review, we present an overview of the information available on the reactivity of various families of therapeutic metallodrugs (mainly anticancer compounds based on Pt, Ru, Au and As) with biomolecules studied by different MS techniques, including high-resolution ESI-, MALDI- and ion mobility-MS among others. Representative examples on the potential of the MS approach to study non-covalent interactions are also discussed. The review is organized to present results obtained on samples with different degrees of complexity, from the interactions of metal compounds with small model nucleophiles (amino acids and nucleobases), model peptides/oligonucleotides, target proteins/nucleic acids, to the analysis of serum, cell extracts and tissue samples. The latter requiring combination of proteomic methods with advanced MS techniques. Correlations between molecular reactivity of metallodrugs and biological activity are hard to establish, but differences in the reactivity of metallodrugs to biomolecules and their different adducts, as revealed by MS methods, may indicate differences in their modes of action. Overall, the knowledge offered by MS methods on metallodrugs speciation is invaluable to establish new rules and define new trends in the periodic table aimed at rationalizing the behavior of metal compounds in complex living systems.

Keywords: metal-based drugs, proteins, nucleic acids, mass spectrometry, metallomics.

Abbreviations

A, adenine
AcO: acetate
Atox1, antioxidant protein 1
BEOV, bis(ethyl-maltolato)oxovanadium(IV)
bipy, bipyridine
bipy^{dmb}, 6-(1,1-dimethylbenzyl)-2,20-bipyridine)
BMOV, bis(maltolato)oxovanadium(IV)
cbdca, 1,1-cyclobutanedicarboxylate
CE, capillary electrophoresis
CID, collision induced dissociation
cisplatin, *cis*-[$PtCl_2(NH_3)_2$]
Cyt c, cythochrome c
CZE, capillary zone electrophoresis
DACH, diaminocyclohexane
dAMP, 2'-deoxyadenosine 5'-monophosphate
dG, 2′-deoxyguanosine
dGMP, 2′-deoxyguanosine 5′-monophosphate
dien, diethylenediamine
DMSO, *S*-dimethylsulphoxide
DTT, dithiothreitol
ECD, electron capture dissociation
EFTEM, energy filtered transmission electron microscopy
en, ethylenediamine
ESI-MS, electrospray ionization mass spectrometry
ETD, electron transfer dissociation
9-EtG, 9-ethylguanine
FASP, fast aided sample preparation
FID, fluorescent intercalator displacement
FRET, fluorescence resonance energy transfer
FT, fourier transform
FT-ICR, fourier transform ion cyclotron resonance
G, guanine
G4, G-quadruplex
GF, gold finger
GSH, glutathione
Gpx, glutathione peroxidase
Hb, haemoglobin
hCtr1, human copper transporter 1
HSA, human serum albumin
HSAB, hard and soft acids and bases
HPLC, high performance liquid chromatography
hTf, human serum transferrin
ICP-AES, inductively coupled plasma absorption emission spectroscopy
ICP-MS, Inductively Coupled Plasma Mass Spectrometry
ICP-OES, inductively coupled plasma optical emission spectroscopy
IEF, isoelectric focusing
Im, imidazole
IM-MS, ion mobility mass spectrometry
In, indazole
IRMPD, infra-red multi photon dissociation
IUPAC, international union of pure and applied chemistry
KCE, kinetic capillary electrophoresis
KP1019, InH[*trans*-In_2RuCl_4]
KP1339, Na[*trans*-In_2RuCl_4]
LA-ICP-MS, laser ablation inductively coupled plasma mass spectrometry
LC, liquid chromatography
LTQ, linear trap quadropole
MALDI-MS, matrix assisted laser desorption ionization mass spectrometry
MP-11, microperoxidase-11

MS, mass spectrometry
MS/MS or MS^n, tandem mass spectrometry
MudPIT, multidimensional protein identification technology
NAMI-A, ImH[*trans*-Im(DMSO)$RuCl_4$]
NHC, *N*-heterocyclic carbine
nESI, nanospray
nLC, nano-liquid chromatography
OmpA, outer membrane protein A
PAGE, 1D polyacrylamide gel electrophoresis gel electrophoresis
phen, 1,10-phenanthroline
PPCs, polynuclear platinum complexes
pta, 1,3,5-triaza-phosphatricyclo-[3.3.1.1]decane
py^{dmb}, 2-(1,1-dimethylbenzyl)-pyridine)
QqQ, triple quadrupole
QToF, quadrupole-time of flight
RAPTA, [(η^6-arene)$RuCl_2$(pta)]
RAPTA-C, [(η^6-p-cymene)$RuCl_2$(pta)]
RAPTA-T, [Ru(η^6-toluene)Cl_2(pta)]
RP, reversed phase
SCX, strong cation-exchange
SEC, size exclusion chromatography
Sec, seleno-cysteine
SOD, superoxide dismutase
SORI, sustained off-resonance irradiation
T, thymine
TCEP, 2-carboxyethylphosphane
terpy, 2,2':6',2"-terpyridine
ToF, time of flight
TrxR, thioredoxin reductase
Ub, ubiquitin
XRD, x-ray diffraction
ZF, zinc finger

1. Introduction

Ancient civilizations discovered centuries ago the potential of metals as pharmaceuticals and, in the last decades, the field of Bioinorganic Chemistry has importantly contributed to the research of new drugs for various diseases. Notably, the first commercially available inorganic compound with therapeutic properties was the arsenic(III)-based drug arsphenamine (Salvarsan®) discovered in 1910 by Paul Ehrlich as anti-syphilis agent. Ehrlich was awarded the Nobel Prize in Physiology and Medicine also for having developed the concept of the "Magic Bullet" according to which it could be possible to kill specific microbes (such as bacteria) that cause diseases without harming the body itself. Interestingly, the structure of arsphenamine has been recently characterized by mass spectrometry methods, showing that it is actually a mixture of trimeric and pentameric scaffolds.[1] Nowadays, a few arsenic drugs are still in use; among them, the greatest clinical success has been the one of arsenic trioxide (As_2O_3, Trisenox®) (Figure 1) in the treatment of hematological cancers, most notably in acute promyelocytic leukemia.[2] Arsenic trioxide almost certainly forms inorganic $As(OH)_3$ in aqueous environment and in this form is transported intracellularly *via* aquaglyceroporin channels due to its similarity to glycerol. Among the successful organoarsenical drugs, melarsoprol (2-[4-[(4,6-diamino-1,3,5-triazin-2-yl) amino]phenyl]-1,3,2-dithiarsolane-4-methanol) (Figure 1) is a *prodrug* currently used as treatment for late-stage east *African trypanosomiasis*, commonly known as sleeping sickness.[3] Melarsoprol is metabolized into the highly reactive melarsen oxide, which irreversibly binds to vicinal sulfhydryl groups causing the inactivation of various enzymes.

Interestingly, in recent years, antimony-based drugs also find applications in the treatment of protozoal diseases. Specifically, pentavalent antimony-containing drugs of Sb(V) with *N*-methyl-D-glucamine such as Pentostam® (sodium stibogluconate) (Figure 1) and Glucantime® (meglumine antimoniate) are the treatments of choice for *Leishmania* infections (leishmaniasis),[4] a disease caused by the protozoan parasite *Leishmania*, from the same family as *Trypanosoma*.

Vanadium complexes have been developed to alleviate insufficient insulin response in diabetes mellitus.[5] Although they may not be able to completely make up for the lack of insulin (as in type 1 diabetes), they can certainly reduce reliance on exogenous insulin, or replace other oral hypoglycaemic agents, in type 2 diabetes.[6] Both bis(maltolato)oxovanadium(IV) (BMOV) (Figure 1) and the ethylmaltol analogue, bis(ethyl-maltolato)oxovanadium(IV) (BEOV), have undergone extensive pre-clinical testing for safety and efficacy,[6] and BEOV has even advanced to phase II clinical trials. Mechanistic studies justify the observed antidiabetic properties in terms of the ability of vanadium complexes to inhibit protein

phosphatases.[7] These significant developments in vanadyl insulin mimetics have prompted further research into the biological applicability of vanadium complexes particularly as anticancer agents and to treat diseases triggered by viruses, bacteria, amoebae and flagellate protozoan parasites. Generally, the active form of vanadium remains elusive, although several studies have reported a number of promising compounds with different geometries and oxidation states.[8]

Specifically concerning cancer treatment, in the late 60s, cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, Figure 1), a platinum(II) coordination complex, revolutionised research in chemotherapeutic agents and is still nowadays considered as the main pioneering discovery in the field of metallodrugs.[9] Since its FDA approval for clinical use in 1978, cisplatin and two other next generation platinum complexes (carboplatin and oxaliplatin) are often used in different chemotherapeutic regimes, generally in combination with organic drugs. The initial studies on the mechanism of action of these platinum(II) anticancer agents identified DNA as the primary target due to the ability of Pt(II) ions to be coordinated by nitrogen atoms of nucleobases.[10] The formation of such adducts (mono- or di-, intra- or inter-strand) modifies the DNA structure, preventing replication and transcription, thus leading to cell apoptosis. Unfortunately, a number of side effects, including toxicity toward certain organs, and the possibility of acquired resistance of cancer cells, reduce platinum(II) compounds efficacy and prompted research towards the development of novel anticancer metallodrugs.[11] In fact, to achieve improved compounds, several strategies have been developed. Thus, while maintaining the platinum centre, rigid bidentate ligands, polynuclear Pt(II) systems (BBR3464)[12, 13] (Fig. 1) and even platinum(IV) prodrugs are examples of the alternatives envisaged with some successes.[14, 15] Afterwards, research in this field has explored other metal-based compounds for their potential in biological applications, among which iron (e.g. ferrocifen (Figure 1)),[16] copper[17] and titanium[18] compounds are particularly representative.[19, 20]

Notably, two ruthenium(III) coordination complexes, namely NAMI-A ImH[*trans*-Im(DMSO)$RuCl_4$] (Im: imidazole) [21] (Figure 1) and KP1019 InH[*trans*-In_2RuCl_4],[22] (In: indazole) entered in clinical trials, with KP1019 still in phase II of the FDA validation processes. Both of these compounds exhibit original properties compared with platinum-based drugs, such as reduced overall toxicity, an ability to overcome the resistance associated with platinum complexes, and in the case of NAMI-A, significant antimetastatic activity.[23, 24] These properties were attributed to the ability of ruthenium-based compounds to be selectively recognised and transported by serum proteins such as transferrin, and to be activated to more reactive ruthenium(II) species at tumour sites, characterized by a more reducing environment than healthy

tissues. More recently, organometallic ruthenium(II) complexes have also been envisaged as therapeutic agents, due to their improved overall stability in physiological environments, but still maintaining the potential of ruthenium to be coordinated to biological targets after hydrolysis of its two chlorido ligands. One of the most representative examples of this class of compounds is the family of antimetastatic and antiangiogenic ruthenium(II) complexes named RAPTA ([(η^6-arene)$RuCl_2$(pta)], pta = 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane, Figure 1) developed by Dyson *et al.*,[25] for which DNA does not appear to be the primary target. In contrast, the family of cytotoxic Ru(II)-arene compounds developed by Sadler *et al.* bearing bidentate diamine ligands and only one chloride ligand (such as [(η^6-p-cymene)Ru(en)Cl], en: ethylenediamine), have been shown to interact with DNA and to exhibit significant anticancer activity on solid tumors *in vitro* and *in vivo*.[26]

The earliest application of gold as therapeutic agent dates far back in ancient centuries, but since the 1920s, various gold(I) thiolate drugs have been used in the clinic to treat mainly rheumatic diseases. In the 1980s, the gold(I) coordination complex auranofin [(2,3,4,6-tetra-*O*-acetyl-1-(thio-kS)- β-D-glucopyranosato)(triethylphosphine) Au(I)] (Figure 1), was found to be highly cytotoxic to tumour cells *in vitro*, opening the way to the evaluation of a range of Au(I) phosphine-based complexes for anticancer applications.[27, 28] In parallel, gold(III) complexes were investigated as potential antitumor compounds, based on the idea that square planar Au(III) d^8 compounds are isoelectronic and isostructural with Pt(II), thus potentially mimicking the cisplatin's activity and ability to react with DNA.[29, 30] Thus, the renaissance of interest for gold compounds as potential anticancer metallodrugs has resulted, in the course of the last decade, in the synthesis of a number of structurally diverse gold(I) and gold(III) species, endowed with sufficient chemical stability and with relevant antiproliferative activities. Initially, the mechanistic studies carried out on cytotoxic gold compounds were generally referred and compared to the behaviour of cisplatin, for which DNA is thought to be the major target. However, from the experimental results collected so far, it has emerged quite clearly that the respective molecular mechanisms are rather distinct and alternative biochemical processes are operative, most likely associated to selective modification of some crucial proteins. In this respect, it is worth noting that gold(I) and gold(III) compounds are known to target, rather strongly and selectively, thiol and imidazole groups of proteins, as well as selenol groups. Moreover, mitochondria, centre of regulation of the intracellular redox balance, have been shown to be likely targets for gold-based complexes of different families.

Overall, metal-based complexes have been demonstrated to possess unique properties and different mechanisms of pharmacological activity with respect to classical organic drugs, thus making them potential candidates for biological applications. However, for most of them, the mechanism of action remains scarcely understood, and while both nucleic acids and/or proteins have emerged as likely targets for these compounds, there is strong need to characterize their reactivity at a molecular level in complex biological systems.

A major issue in the field of therapeutic metallodrugs development is that these compounds are often *prodrugs* in need to undergo activation processes to exert their pharmacological effects (e.g. cisplatin is activated by hydrolysis inside cancer cells). Therefore, studies of metallodrug *speciation* are necessary to "fine-tune" the stability of the metal complexes while maintaining their biological activity and reducing their side effects. It is worth reminding that, in 2000, an IUPAC report recommended that the term "speciation" of an element should be used to indicate species distribution, and "speciation analysis" be used to indicate the analytical activity of identifying and/or measuring the quantities of one or more chemical species in a sample.[31] Noteworthy, the broad and often unspoken definition of speciation generally used in coordination chemistry includes metal complexes that can undergo reactions in solution resulting from hydrolysis, redox changes, ligand coordination, and geometric isomerization reactions.[32]

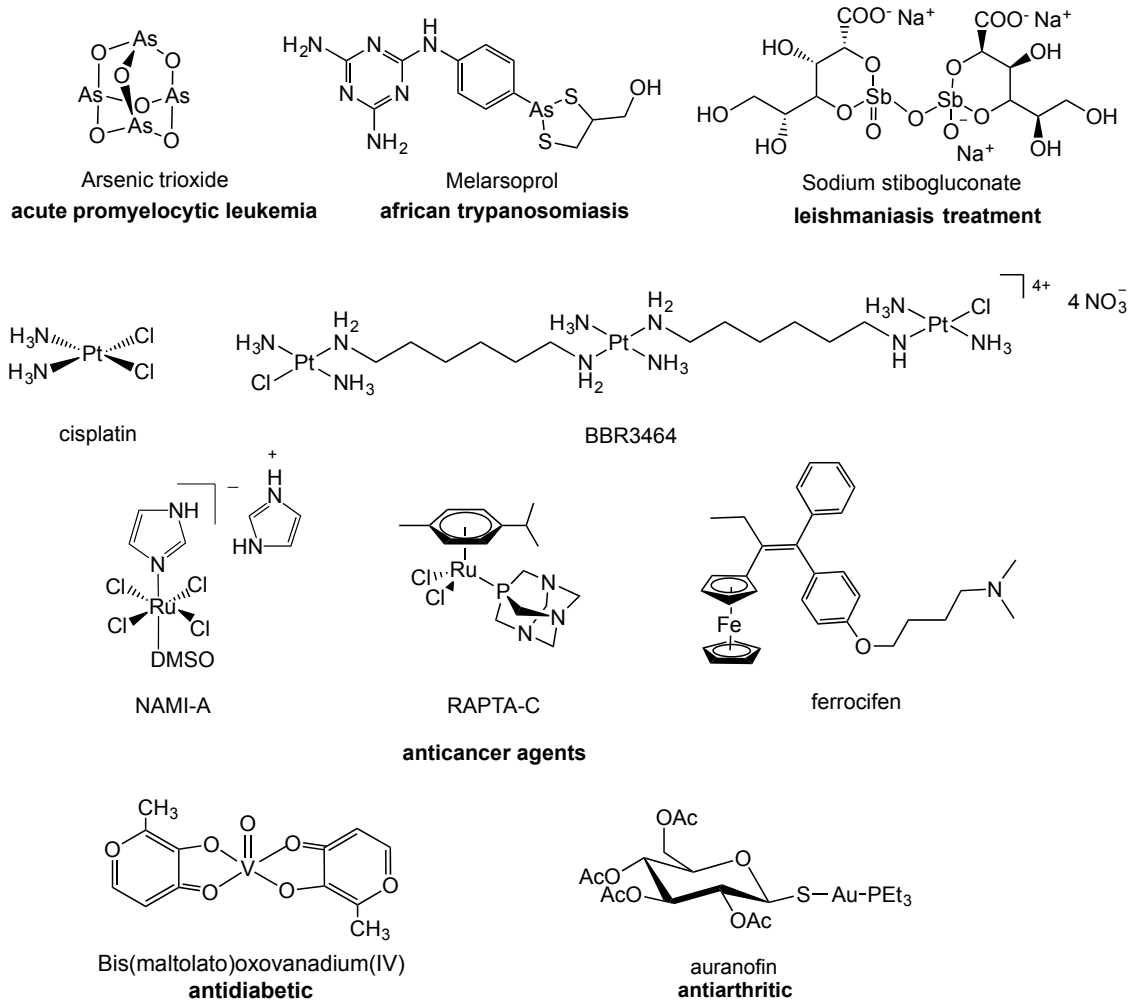


Figure 1. Therapeutic metal compounds (marketed or experimental).

Nowadays, the study of the metallodrugs speciation and their reactivity with biological nucleophiles may take new and considerable advantages of the availability of sophisticated and high-resolution analytical methods. In this context, mass spectrometry techniques are valuable investigational tools to provide information on the biological interactions of metal-based compounds at various levels. For example, quantitative MS methods such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and LA-ICP-MS (LA = Laser Ablation) can quantify metal content in tissues/cells, as well as provide information of the biodistribution of the metal in different organs, and of its affinity for binding to certain cellular components (e.g. proteins). With the aim of achieving direct monitoring of real-time biomolecule-mediated metabolism of anticancer agents, the use of hyphenated (coupled, tandem) techniques, that combine chromatographic separation to ICP-MS methods, has been of great importance to simplify the original biological sample. Thus, in terms of separation techniques, high performance liquid chromatography (HPLC)[33] and capillary zone

electrophoresis (CZE),[34, 35] as well as size exclusion chromatography (SEC),[36] were proposed for the study of different metal-based drugs, to assess kinetic constants of hydrolysis and subsequent reactions with a variety of physiological targets.

In parallel, molecular MS methods such as Electrospray Ionization Mass Spectrometry (ESI-MS) and Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) are the most appropriate techniques to analyse the precise interactions of metallodrugs with biomolecules, including DNA and proteins, at a molecular level (Figure 2).

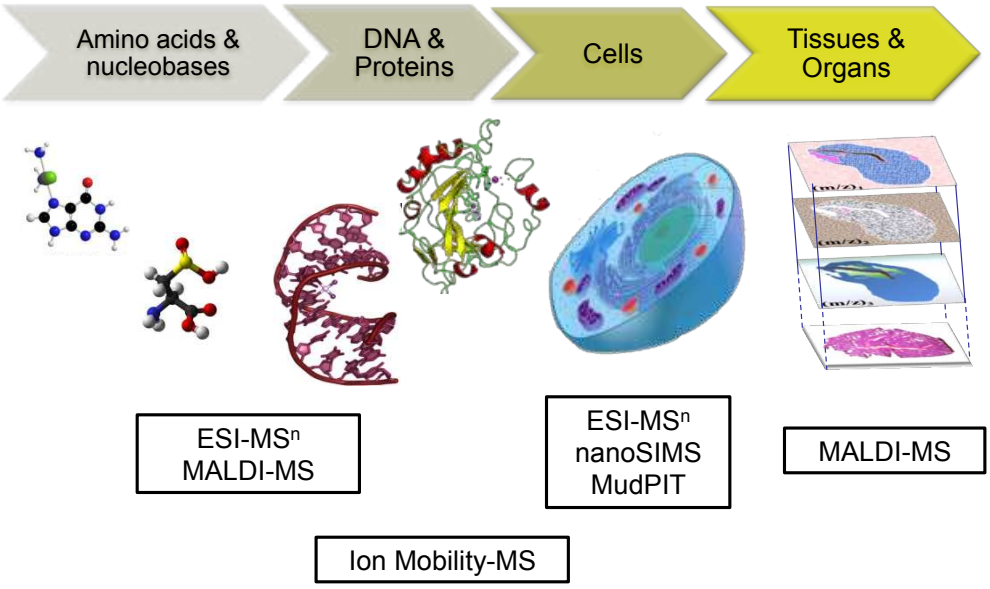


Figure 2. Representative examples of the molecular MS tools used to study metallodrugs speciation in simple to more complex systems.

Briefly, while ESI generates singly to multiply charged ions by adduct formation with protons, alkali metal ions or by deprotonation, resulting in a characteristic charge envelope for peptides, proteins and oligonucleotides, MALDI yields mostly singly charged pseudo-molecular ions of analytes. In terms of advantages, while ESI allows analysis of both covalent and non-covalent interactions and can be easily hyphenated to separation techniques (e.g. capillary electrophoresis (CE) and liquid chromatography (LC)), MALDI can detect only covalent or pseudo-covalent adducts and the system cannot be directly combined to chromatographic methods. For a more technical description of the MS methods applied to the investigation of metallodrugs we refer the reader to a recent tutorial review.[37] Often the selection of the technique is done accordingly to the molecular weight of the biomolecule under investigation: for example MALDI-MS, based on the bombardment of the sample pre-mixed with an absorbing matrix with a laser, is well suited for the analysis of high molecular weight (> 50 KDa) and non-volatile compounds.

In terms of mass analysers, the choice often depends on the application and required resolution, but commonly also on the cost. While triple quadrupole instruments are relatively cheap, they are normally used for quantification in metabolomics and targeted proteomics approaches. The analysis of high molecular weight samples is mainly performed with more expensive quadrupole-time of flight (QToF) or Fourier Transform (FT)-based Orbitrap and FT-ion cyclotron resonance (FT-ICR) mass spectrometers, which are also used in proteomics and other applications requiring isotopic resolution and high mass accuracy.

Interestingly, while mass spectrometry is a vital tool for molecular characterization, the allied technique of ion mobility is enhancing many areas of (bio)chemical analysis. Ion mobility separates ions (from small molecules up to mega Dalton protein complexes) based on their differential mobility through a buffer gas. Therefore, ion mobility mass spectrometry (IM-MS) can act as a tool to separate complex mixtures, to resolve ions that may be indistinguishable by mass spectrometry alone, or to determine structural information (for example rotationally averaged cross-sectional area), complementary to more traditional structural approaches.[38] Finally, IM-MS can be used to gain insights into the conformational dynamics of a system, offering a unique means of characterizing flexibility and folding mechanisms.

In order to determine the binding sites of metallodrugs in proteins, peptides or oligonucleotides strands, ESI-MS coupled to tandem mass spectrometry techniques have been developed (Figure 2). Tandem mass spectrometry, also called MS/MS or MS^n, involves multiple steps of mass spectrometry experiments, giving rise to different types of fragmentations can occur. In a MS/MS experiment, ions are firstly formed in the ion source and separated according to their *m/z* (m: mass; z: charge) ratio. Then, a selection of precursor ions can be chosen and fragment ions (product ions) can be generated by different techniques, such as CID (Collision Induced Dissociation), IRMPD (Infra-Red Multi Photon Dissociation), ETD (Electron Transfer Dissociation) or ECD (Electron Capture Dissociation). It is worth mentioning that ToF analyzers have the advantage to work on a wide mass range, but have limited MS^n capabilities, which may also influence their choice in certain studies. Overall, tandem MS techniques allow the exploration of the targets of metal complexes at a molecular and even atomic level by fragmentation techniques, using top-down (fragmentation of intact analytes) or bottom-up (enzymatic digestion of the analyte prior to fragmentation by MS and MS^n techniques) approaches. Such information is crucial and must be integrated into a set of data from different experimental approaches such as bioanalytical, biochemical, biophysical and biological studies. In fact, it should be noted that MS methods are not deprived of limitations: specifically, data analysis requires care in extrapolating gas-phase data to solutions and physiological conditions, since

artefacts induced by the ionization process may occur during metal adduct formation. This is particularly true when MS methods are applied to investigate non-covalent and outer-sphere interactions based on electrostatic, hydrogen-bonding and π-π stacking, between metal compounds and their targets. In this respect, while MS has the advantage of evidencing such "labile" interactions in comparison with other methods, several control experiments should be performed to validate the data interpretation.

Finally, among the most advanced methods to investigate metallodrug's speciation in cellular samples, the so-called Multidimensional Protein Identification Technology (MudPIT) is highly valuable. This technique is based on the hyphenation of 2D liquid chromatography (reversed phase and strong cation-exchange (SCX)) and ESI-MS^n and allows up to 1500 proteins to be analysed in 24 h. Thus, MudPIT allows the molecular study of complex systems exposed to metallodrugs, but the technique also suffers from several limitations.[37] For example, the metal of interest must have a specific isotopic pattern that allows undeniable identification of the fragmented proteins and peptides. Furthermore, the binding sites of the metals to their biological targets should be stable enough to resist tandem mass spectrometry fragmentation, tryptic digestion and/or SCX separation (achieved with strongly acidic conditions) and to prevent intra- or inter-molecular transfer to alternative sites during the analysis that would lead to misinterpretation of the results. Finally, the sensitivity of the spectrometer must be very high to allow the detection of peptides in the fmolar range, corresponding roughly to a hundred copies per cell.[39]

In this context, this review aims at summarizing the results on therapeutic metallodrugs' speciation obtained *via* molecular MS methods (mainly ESI- and MALDI-MS), in some cases combined to hyphenated techniques (e.g. LC) or run in parallel to quantitative techniques (mainly ICP-MS), and at providing an overview of the state-of-the art knowledge of the therapeutic metal complexes' interactions with biomolecules and biological components as provided by these methods. The information is organized in the various sections starting from the results obtained with model biomolecules and nucleophiles, to proceed with those regarding pharmacological targets, and towards increased level of complexity of the samples (serum, cell extracts and tissues).

2. Interactions of metallodrugs with model systems

Before evaluating the targets of metallodrugs in complex biological systems, mass spectrometry has been used to analyse the interactions within simpler model systems, to give a proof-of-concept of the technique, as well as information on the molecular and even atomic interactions established by metal complexes with

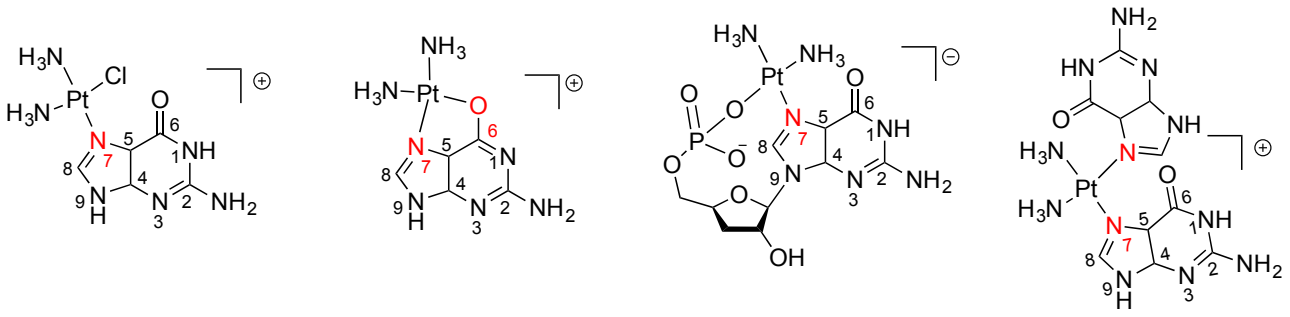
macromolecules. ESI-MS being a “soft” ionization method in avoiding the use of a matrix component to induce formation of sample ions in the gas phase, is particularly adapted to obtain conformational and binding site information with high sensitivity.

2.1 Mass spectrometry studies of nucleobases and amino acids

As most of the metal-based complexes with anticancer activity described so far target either DNA or proteins or both, the simplest systems used to analyse the interaction of a drug with these targets at a molecular level consist of amino acids, nucleobases and nucleotides. Below we describe the most comprehensive studies on anticancer compounds based on platinum, ruthenium and gold ions.

Platinum complexes

The antiproliferative properties of cisplatin and other platinum-based anticancer agents are acknowledged to be the result of interactions of the platinum(II) ions mainly with two adjacent guanines (or guanine-adenine) in duplex DNA. Such interactions have been extensively described using a variety of techniques (NMR, circular dichroism, electrophoresis, X-ray diffraction etc.).[40, 41] As far as mass spectrometry is concerned, different ionization methods have been used to characterise the nature of the binding sites, directly or often implemented with separation techniques such as capillary electrophoresis (CE) or HPLC, in order to isolate the different mono- and bis-adducts and to gain insights on their kinetics of formation as well as their quantification.[42] Thus, in early studies, CE and CZE-ESI-MS were used to study the nature of the binding between guanosine and the dGMP (2′-deoxyguanosine 5′-monophosphate) model residue and cisplatin.[43, 44] The major platination site of cisplatin on guanine is known to be the N^7 atom (Figure 3, Structure A), resulting in the formation of 1,2-d(GpG) intra-strand cross-links (dG = 2’-deoxyguanosine, p = linking phosphate group) and, thus, leading to inhibition of DNA replication.[45]



A **B** **C** **D**

Figure 3. Proposed structures of the mono- and bis-chelates for the reaction between cisplatin and dG and dGMP.

Participation of the O^6 atom of guanine in the binding of cisplatin, resulting in the formation of a bidentate O^6-N^7 adduct (Figure 3, Structure B), was also hypothesized to occur.[46] However, no structural information could be obtained to confirm this hypothesis so far. NMR spectroscopy experiments have actually discredited the implication of the O^6 atom in platination of guanine residues,[47] but rather indicated the possibility of N^7- αPO_4 macrochelate formation involving the phosphate group of nucleotides in Pt binding (Figure 3, Structure C).[48] Using CE coupled to ESI-MS, Jaehde *et al.* investigated the binding of cisplatin to dGMP.[43] Using an equimolar amount of cisplatin and dGMP, the main adducts were identified as the bis-adduct (N^7-dGMP)$_2$Pt(NH$_3$)$_2$ (Figure 3, Structure D), as well as the mono-adducts [(dGMP)Pt(NH$_3$)$_2$Cl]$^+$, [(dGMP)Pt(NH$_3$)$_2$OH]$^+$ and [(dGMP)Pt(NH$_3$)$_2$]$^+$, respectively. Moreover, the results suggested that O^6-N^7 chelation is a possible coordination mode of platinum(II) complexes with DNA purine bases (and derivatives of them). In fact, upon incubation of cisplatin with hydrogen peroxide to potentially stabilise the O^6-N^7 chelate as a Pt(IV) specie, an adduct corresponding to [(O^6-N^7-dG)Pt(NH$_3$)$_2$(OH)$_2$]$^+$ was indeed detected.[43] However, it should be noted that no further evidence of this type of adducts has been provided so far by other methods, and influence of the gas-phase nature of the ESI-MS experiment cannot be excluded. Furthermore, when cisplatin was incubated with dG (deoxyguanosine), an adduct corresponding to [(dG)Pt(NH$_3$)$_2$]$^+$ was also observed, excluding the hypothesis of the formation of the N^7-αPO$_4$ macrochelate.

Notably, the reactivity of cisplatin towards dGMP was found to be highly dependent of the nature of the buffer system.[44] In fact, in a systematic approach, Keppler *et al.* studied the binding of cisplatin (1 mM) to dGMP (2 mM) in different incubation conditions of relevance to biological systems, employing capillary zone electrophoresis (CZE) combined to UV-Visible and ESI-MS detectors. While adduct formation (mainly [Pt(NH$_3$)$_2$(dGMP)$_2$-3H]$^-$) was found to be rapid in pure water, the use of buffers such as carbonates or phosphates significantly reduced the reactivity of cisplatin, in some cases with detection of sequestration of the platinum ions by the phosphate buffer anions.[44] Notably, carbonate buffer had a major impact on the binding, though no coordination to the metal centre was detectable with the selected methods.

Based on these studies, it becomes apparent that the binding of cisplatin to dGMP, and most likely towards any biological target, strongly depends on the buffer composition. This factor should be considered when experiments under pseudo-physiological conditions are conducted. Furthermore, adducts formed with

buffers/salts might have a completely different biological profile in *in vitro* or *in vivo* settings than the parent compounds.

The degradation pathway of the main adducts characterised between cisplatin and DNA purine bases ($[G+Pt(NH_3)_2Cl]^+$, $[G+Pt(NH_3)Cl]^+$, $[A+Pt(NH_3)_2Cl]^+$, $[A+Pt(NH_3)Cl]^+$) has also been studied by fragmentation using CID.[49] In both cases, the loss of either one or two NH_3 molecules has been observed, as well as of HCl. In the case of the adenine-Pt adducts, the MS/MS spectra also revealed the presence of free protonated adenine with loss of the Pt adduct, which isn't observed in the case of guanine, and confirms the strongest binding of cisplatin to guanine bases over adenine ones.[49] Further tandem MS experiments have been reported to compare the interactions of cisplatin with adenine and guanine combining theoretical methods and CID-MS/MS techniques.[50] The results showed that cisplatin was potentially able to bind the dGMP model DNA base on the N^7, N^3 and O^6 atom, whereas the anchoring sites on dAMP (2'-deoxyadenosine 5'-monophosphate) were suggested to be N^7, N^3 and N^1.

Oxaliplatin has also been studied for its ability to bind all four DNA nucleobases by linear ion trap ESI-MS techniques.[51, 52] When incubated individually, all nucleobases bound oxaliplatin in a mono- or bis-adduct fashion, with the initial platinum complex losing its oxalate ligand while preserving the DACH (DiAminoCycloHexane) moiety. Furthermore, when oxaliplatin was incubated with a mixture of all four bases, a clear preference for adenine and guanine residues was noticed, the main adducts on the spectra being identified as Pt-A, Pt-G, Pt-AA and Pt-AG. In a significant lesser extent, adducts corresponding to Pt-GG, Pt-C and Pt-T have been observed as well. MS^2 experiments using CID fragmentation technique have been described to study the degradation pathway of the main adducts, showing that the di-adducts release a DNA base, then H_2 and ammonia.[51]

Keppler *et al.* also performed a systematic study of the binding behaviour of several platinum-based anticancer drugs towards the 5'-GMP (5'-guanosine monophosphate) DNA model nucleobase in presence of sulfur-containing amino acids using CE-ESI-MS ion trap techniques.[53] In the absence, as well as in the presence of cysteine or methionine (co-incubated in a 1:1:1 5'-GMP/platinum drug/amino acid ratio), the reactivity of the metallodrugs towards 5'-GMP followed the order: cisplatin > oxaliplatin > carboplatin. As expected, the nature of the adducts was found to be highly dependent on the nature of the ligands on the initial Pt complex. For the complexes bearing more labile ligands such as cisplatin and carboplatin, the adducts $[Pt(NH_3)_2(GMP\text{-}N7)_2]^{2-}$ reacted with methionine to form the new products $[Pt(NH_3)(GMP\text{-}N7)(L\text{-}Met\text{-}S,N)]^-$. The bidentate DACH ligand of oxaliplatin was shown to prevent coordination of both the DNA base

and methionine at the Pt(II) centre, but rather favoured the amino acid reaction upon loss of the oxalate ligand. Cysteine has been found to have no or very poor influence on the reaction between 5'-GMP and the Pt(II) complexes except for oxaliplatin. In this latter case, a competitive binding between cysteine and 5'-GMP has been observed, supported by the detection of $[Pt(DACH)(L\text{-}Cys\text{-}S,N)]^+$ adducts.[53] Overall, it was concluded that the stability of the DACH platinum fragment prevents the formation of mixed-ligand adducts as it is the case for platinum complexes with ammine ligands. The observed differences in reactivity may relate to important differences in the mechanisms of action of the various Pt(II) drugs.

Apart from the purine bases of DNA, cisplatin as well as other platinum-based derivatives were studied for their ability to bind to single amino acids. In general, high affinity for binding to methionine residues has been confirmed by ESI-MS.[54, 55] Another example reported on the ability of glutamic acid to bind to a $[Pt(DACH)(OH_2)_2]^{2+}$ complex to form adducts of the kind [L-glutamic acid + Pt(II) + DACH]$^+$, as determined by ESI-MS experiments and confirmed by other analytical methods.[56] In any case, the reported studies confirm the bidentate coordination to Pt^{2+} by the amino acid residues (acting as chelating agents), occurring *via* metal binding to side-chains' residues and/or to N or O atoms of the amide backbone.

Ruthenium complexes

Similarly, Ru-based anticancer agents have been evaluated for their reactivity towards both amino acids and nucleobases by MS techniques. Thus, Sadler *et al.* investigated the interactions of $[(\eta^6\text{-biphenyl})RuCl(en)]^+$ (en: ethylenediammine) complexes with Cys, Met and His isolated by HPLC and characterized by LC-ESI-MS and NMR spectroscopy, showing the evolution of the nature of the adducts, from mononuclear after a few hours to dinuclear after 24 h incubation.[57, 58] The study also highlighted the preference of Ru(II) for binding to cysteine residues.

Furthermore, the possible influence of the biological reducing agents (such as glutathione inside cells or ascorbic acid found in the blood stream) on the binding of KP1019, a Ru(III) complex, to nucleotides has been studied by CE-MS. The results highlighted the improved ability of the Ru(II) center to bind to the model nucleotide 5'-GMP after reduction by glutathione, confirming the so-called 'activation by reduction' process suggested for Ru(III) prodrugs.[34, 59, 60]

Organometallic RAPTA complexes have been investigated for their binding to DNA nucleobases by ESI-MS, and it was found that in physiological media, after hydrolysis of one of the chlorido ligands, the complexes were highly reactive forming mainly mono-adducts of the general formula $[(\eta^6\text{-arene})RuCl(pta)(DNA\ base)]^+$

with 9-ethylguanine, guanosine, adenosine and inosine models, while scarcely reactive towards other bases such as cytidine, thymidine and uridine.[61, 62] The structure of the adducts and their relative stability was also assessed by tandem MS experiments.[62] RAPTA-based complexes have also been studied by ESI-ToF-MS for their interactions with the amino acids histidine and methionine, and similar results as described above showed their tendency to form mono-adducts with substitution of one of the chlorido ligand (most likely already exchanged with aqua or hydroxo ligand in aqueous media) without loss of the arene, even in presence of other biological nucleophiles.[59]

Gold complexes

Concerning anticancer gold complexes, the reaction mechanism of different gold(III) coordination compounds bearing N-donor ligands (e.g. bipyridine, terpyridine, etc.) with amino acids and peptides and the structural characterization of the resulting gold adducts have been investigated *via* different methods, including mass spectrometry techniques.[63] In addition, the reactivity of gold(III) compounds/ions with model amino acids and peptides has also been explored, in some cases allowing structural characterization of the resulting adducts.[64] Notably, Djuran *et al.* have studied the mechanism of oxidation of amino acids (glycine, methionine, histidine, cysteine, etc.) by Au(III) ions ($AuCl_4^-$ and $[Au(en)Cl_2]^+$ by various techniques, including MS, at acidic and physiological pH.[64] Using a multi-instrumental approach, Au(III) complexes were found in general to be all reduced to Au(I) and Au(0) species. Specifically, Au(III) compounds were found to induce oxidation of glycine and alanine, causing their deamination and subsequent decarboxylation. On the other hand, histidine was found able to form initially Au(III) species with one (in the case of $Au(en)Cl_2$) or two (in the case of $HAuCl_4$) amino acids bound at low pH. At higher pH, a fast oxidation of histidine was detected, accompanied by reduction of the Au(III) compounds. The reaction between cysteine and Au(III) compounds gave rise to the formation of cystine and Au(I) ions, the latter stabilized by the excess of cysteine. Finally, methionine was found able to form short-lived Au(III) adducts, then evolving into methionine sulfoxide and Au(I) reduced species.

So far, fewer studies have been available concerning organometallic cyclometalated gold(III) compounds and their reactivity with biomolecules, which highlights the differences and possible advantages in developing organometallic compounds with respect to classical coordination complexes. Recently, Casini, Meier *et al.* reported on the investigation of five Au(III)-based anticancer agents, either organometallic (cyclometallated C,N,N- and C,N- derivatives) or coordination complexes, by ESI-IT MS techniques for their

binding preferences to amino acids in a competitive experiment.[65] The His, Cys, Met, Glu, Se^{Me}-Cys and Se-Cys residues have been selected for their proposed involvement in binding to Au(III) ions.[29, 64] The general trend drawn from this study suggests that cyclometallated Au(III) complexes are able to bind amino acids residues such as Se-Cys, Cys and in some cases His, remaining in the oxidation state +III, whereas the coordination Au(III) complexes undergo preferably reduction of the Au(III) centre to Au(I) or Au(0) and, in some cases, oxidation of the cysteine residues to form cystine (Figure 4). Interactions with nucleobases and nucleotides have also been described by ESI-IT MS. In most cases, the organometallic Au(III) species show no or very limited interaction with the 9-ethylguanine model, whereas the coordination complexes were observed to form mono-adducts after 24 h incubation, with both Au(I) and Au(III) oxidation states ($[LAu^{III}(9\text{-EtG})\text{-}2H]^+$ and $[LAu^{I}(9\text{-EtG})]^+$). Nucleobases have also been used to evaluate the effect of the additional negative charges of the phosphate skeleton by ESI-IT MS in negative mode. Interestingly, the organometallic Au(III) complexes formed nucleotide adducts with both ATP and GTP, whereas Au(III) coordination complexes were unable to form any kind of adduct with the nucleobases.

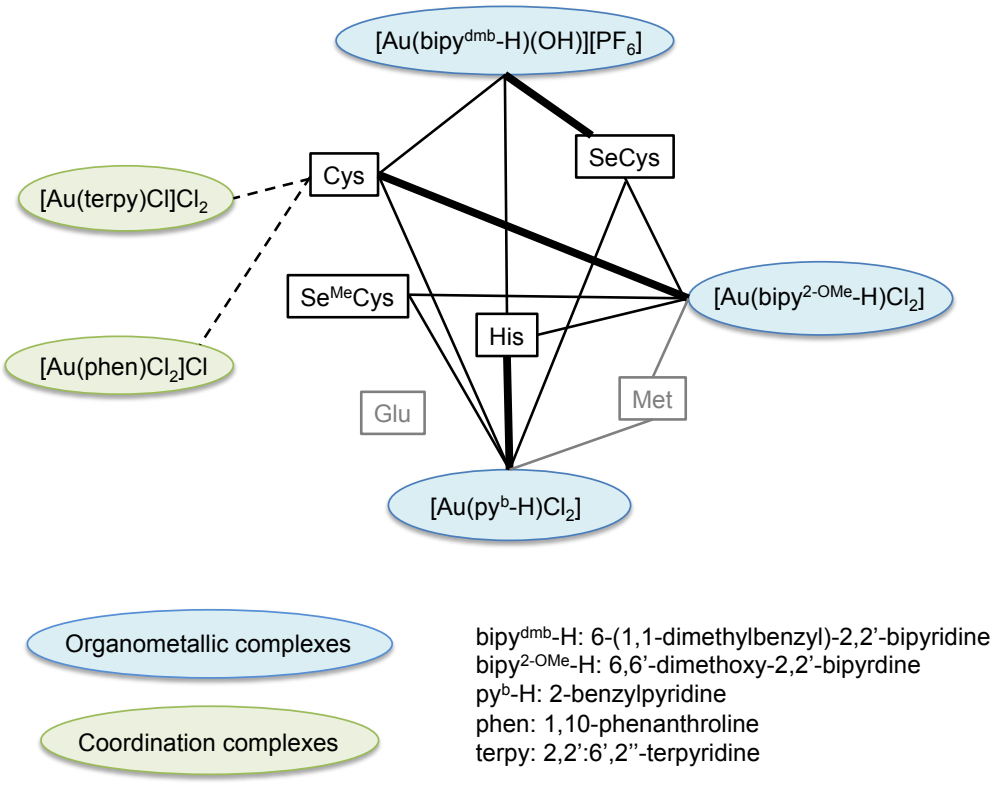


Figure 4. Structures of gold(III) complexes investigated by ESI-MS and interaction network of the compounds with model amino acids and preferences for adduct formation. Circles denote Au^{III} compounds, boxes denote amino acids, straight lines indicate detected Au^{III}-amino acid adducts, and dashed lines indicate indirect evidence for interaction. Gray boxes and lines indicate Au^{III}-amino acid adducts that were not observed in the presence of a selenium-containing amino acid. Adapted from ref.[65]

2.2. Mass spectrometry analyses of oligonucleotides and peptides

To achieve better understanding of the molecular targets of metallodrugs in physiological environment, and to consider the influence of moderately complicated model systems towards the reactivity of the metal complexes, single- and double-stranded DNA oligonucleotides, as well as a number of peptides of 3 to 14 amino acids length (e.g. angiotensin, substance P, bombesin, glutathione) have been studied by MS techniques.

Platinum complexes

Cisplatin and other platinum-based complexes have been extensively described for their binding properties to oligonucleotides (short fragments of DNA) by MS.[52, 66, 67] The preferential binding of cisplatin to the purine-N^7 of DNA bases, leading to the formation of intra-, inter-strand or mono-adducts has been demonstrated using different techniques, in accordance with the results obtained on single nucleobases.

A “bottom-up” approach, consisting in enzymatic digestion coupled to MS, was used to demonstrate the nature of the adducts formed by incubation between cisplatin and oligonucleotides of different contents and lengths (single stranded).[68, 69] For example, Chottard *et al.* used enzymatic digestion coupled to ESI-MS and MALDI to determine the rate constants of platination for the hydrolysed cisplatin species $[Pt(NH_3)_3(H_2O)]^{2+}$] and the ratio of the different adducts on the octapeptide d(TTGGCCAA). As expected, the platination was much faster in water rather than in 0.1 M $NaClO_4$ buffer, but still featuring the same selectivity of the Pt(II) ions for the 5’-guanine residue.[69] In addition, 8- and 16-mer oligonucleotides containing GA, AG and GG sequences have been used to study the nature and preference of cisplatin’s adducts formation by enzymatic digestion followed by ESI-MS. All three model DNA sequences can form adducts with cisplatin; however, GG and AG sequences have appeared as favourite binding sites.[68]

It is worth mentioning that, in general, the “bottom-up” approach is hampered by the use of enzymes such as exonucleases, which cannot cleave most oligonucleotides that have been modified by metallodrugs and might consequently lead to an incomplete set of data. Moreover, binding of Pt complexes to the enzymes in the sample may also interfere with DNA adduct formation. Instead, “top-down” approaches, in which the fragmentation occurs on intact analytes, have been used to study oligonucleotides-platinum drugs adducts by different MS fragmentation techniques.[70, 71] The advantages of such studies are that the analyses can be performed under almost native conditions and, in most cases, the complete sequence of

double-stranded oligonucleotides can be determined. For instance, Dyson *et al.* described the use of FT-ICR MS in the negative mode to probe the interactions of cisplatin with 13-mer double-stranded DNA oligonucleotides, which allowed accurate localisation of the preferential platinum binding sites and determination of the binding kinetics.[72] Furthermore, selective fragmentation using MS/MS with CID and IRMPD revealed that the binding starts initially with formation of intermediate $[Pt(NH_3)_2Cl]^+$ species, followed by generation of bifunctional $[Pt(NH_3)_2]^{2+}$ adducts. Finally, further platination can lead to bis-$Pt(NH_3)_2$ adducts. Tandem MS experiments also revealed that cisplatin can form up to 3:1 Pt/oligonucleotide adducts, with the sequences GG and GTG clearly preferred. To confirm the preference of cisplatin for guanine residues even in presence of large amounts of adenine and thymine, and to establish the favourite binding sites for oxaliplatin and carboplatin, MS/MS experiments using CID were also described.[73] As expected, the results showed that guanine remains the preferred binding site in all cases.

The smallest peptide reported for its ability to react with cisplatin is glutathione (GSH), which is a sulfur-containing tripeptide of sequence Glu-Cys-Gly with a gamma peptide linkage between the carboxyl group of the glutamate side chain and the amine group of cysteine (anchored by classical amide bond to glycine). GSH is an essential intracellular reducing agent that maintains the cellular redox balance and can form disulfide bonds in physiological media. According to the HSAB theory, the sulfur atom can theoretically easily coordinate to metal complexes. In fact, GSH is present in cells at various concentrations (0.5-10 mM)[74] and is believed to induce detoxification of platinum and ruthenium-based metallodrugs.[75] It has therefore been widely used in competitive MS experiments with metallodrug-biomolecule adducts. A few examples of such competition studies will be reported later in this chapter.

In early studies, Zhao *et al.* reported on the use of ESI-MS associated with CID to determine the nature of potential GSH-cisplatin adducts.[76] The main adduct on the ESI-MS spectrum was identified as $[2GSH-2H+2Pt+4NH_3]^{2+}$. This adduct was then subjected to CID fragmentation to determine the binding sites. The four NH_3 ligands were easily released, which most likely indicate their *trans* positions relative to sulfur ligands, giving rise to the hypothesis that the adduct consists in a dinuclear four-membered Pt_2S_2 ring (Figure 5), as similarly observed with oxaliplatin.[77] In order to determine the ability of platinum to form adducts of this kind with other model peptides, two other small peptides (Met-Arg-Phe-Ala and Ac-Met-Ala-Ser) were incubated with cisplatin in the same conditions.[76] Using CID fragmentation, Pt was observed to form a mono-adduct using the three donor atoms of methionine (S, NH_2 and NH) and still bearing one of its

initial NH_3 ligands, or when the methionine residue was acetylated, a $[Pt(NH_3)_2]^{2+}$ specie coordinated *via* the S and NH residues only (Figure 5).

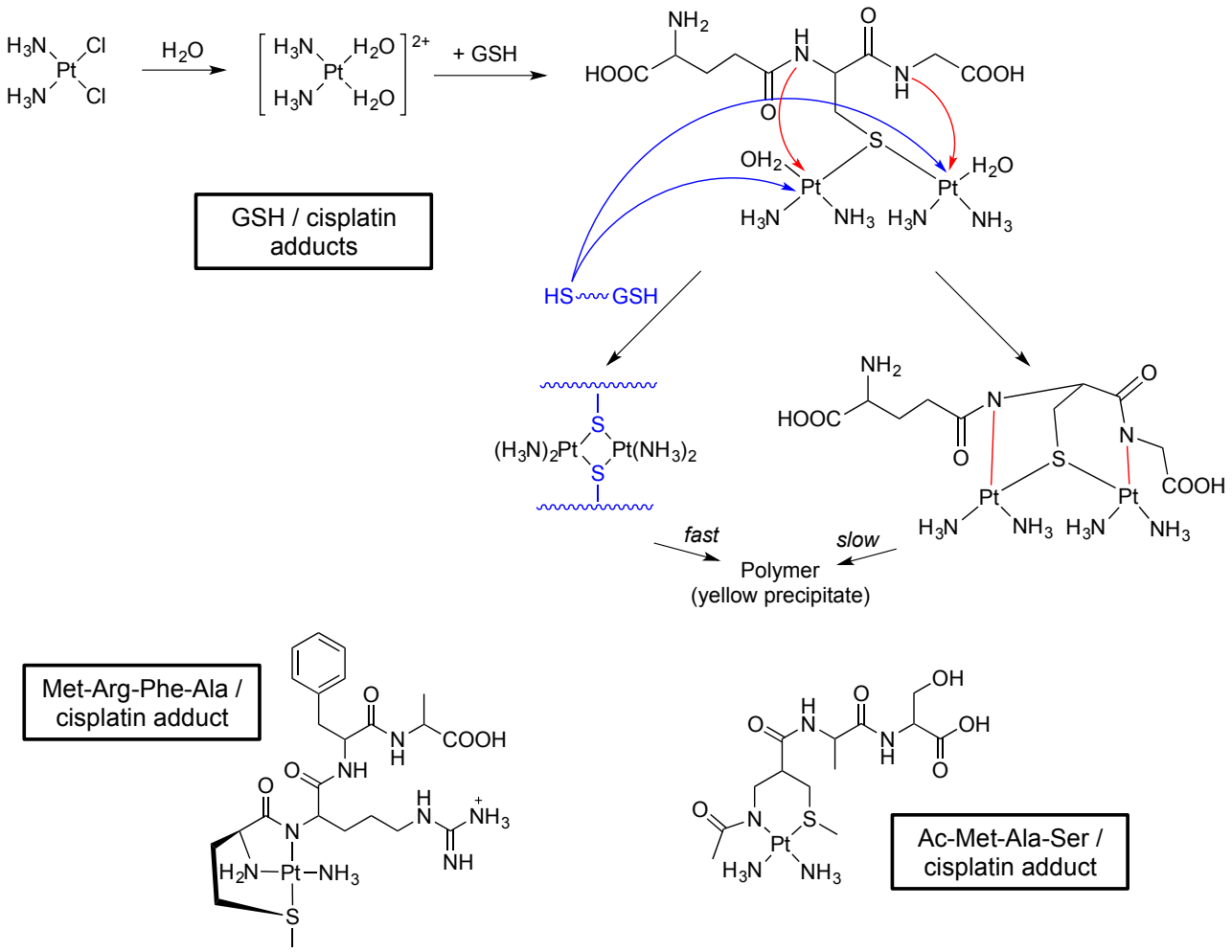


Figure 5. Scheme of the adducts identified by MS^n by incubation of cisplatin with glutathione and the peptides Met-Arg-Phe-Ala, Ac-Met-Ala-Ser. Adapted from ref [76].

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) techniques using both CID and ECD have been employed to locate the binding sites of cisplatin on model and commercially available peptides such as substance P (11 amino acids), angiotensin (7 amino acids) and bombesin (14 amino acids).[78, 79] A combination of different fragmentation techniques (CID and ECD) as well as ion mobility mass spectrometry (IM-MS) allowed the determination of the sulfur atom of the methionine residue of substance P as the favoured binding site of cisplatin, whereas the His residues of angiotensin II and bombesin appeared to be preferred.[78-80]

Tandem mass spectrometry techniques have been used to characterize cisplatin interactions with peptides representing fragments of proteins of interest. For instance, Microperoxidase-11, a peptide

fragment of cytochrome c, has been used to evaluate the nature of the adducts of cisplatin.[81] While ESI-MS technique has been able to evaluate the presence of a mono-adduct [MP-11+Pt$(NH_3)_2(H_2O)]^+$ upon incubation, tandem MS technique using CID and IRMPD fragmentations allowed the determination of the platinated sequences of amino acids of MP-11 (^{1}Met-Gln^2 with CID and ^{1}Met-Gln-Ile-Phe^4 with IRMPD) and the most likely binding site (N-terminal ^{1}Met) in accordance with the HSAB theory.

An interesting case study recently compiled in a review by Komeda, Farrell *et al.* is the one related to the mechanistic investigation of anticancer substitution-inert polynuclear platinum complexes (PPCs), such as BBR3464 (Figure 1), which bind to DNA through the "phosphate clamp", a discrete mode of DNA-ligand recognition distinct from the canonical intercalation and minor-grove binding.[82] Following incubation of some PPCs with duplex oligonucleotides (ca. 5000 Da each), ESI-MS spectra coupled to CID fragmentation were recorded and showed that, even though the interactions between the complexes and the DNA strands were non-covalent in nature, they were strong enough to be observed in the gas phase during the analysis, and confirmed that PPCs do significantly stabilise the duplex structure of the oligonucleotide strands.[82]

Ruthenium complexes

Ruthenium-based compounds have also been characterised for their reactivity towards oligonucleotides and peptides using MS techniques. For example, the reactivity of RAPTA-C [(η^6-p-cymene)$RuCl_2$(pta)] (Figure 1) towards the sulfur-containing tripeptide glutathione has been evaluated using FT-ICR-ESI-MS techniques.[83] After 1 h incubation, new adducts were detected, corresponding mainly to [Ru(*p*-cymene)(GS)]$^+$ and [Ru(*p*-cymene)(GS)(pta)]$^+$ species. However, after 24 h incubation, the mass spectrum changed dramatically, the most abundant peaks being assigned as non-ruthenium containing species indicating further fragmentation of both RAPTA-C and GSH.

Sadler *et al.* reported the use of ultra-high resolution FT-ICR MS techniques to study the interaction of [(η^6-biphenyl)(en)RuCl]$^+$ complexes with peptides such as angiotensin and bombesin, as well as glutathione.[84-86] In the cases of angiotensin and bombesin, the fragmentation was performed using CID, while additional data were obtained using ECD. The results showed that the primary binding sites of such complexes were methionine and histidine residues, with phenylalanine being a potential secondary site (potentially *via* π-stacking interactions with the ligands of the Ru complex). The reaction between the same Ru complex and the tripeptide GSH was monitored using nLC-FT-ICR MS in combination with ^{18}O-labelling.[86] Glutathione was observed to form mainly a covalent adduct with the complex of the type [{(η^6-

biphenyl)Ru}$_2$(*S*-GS)$_3$]$^{2-}$, which is then very prone to oxidation of the GS ligands to give rise to clusters of multinuclear complexes accompanied by reduction of the ruthenium centres (di-ruthenium and tetra-ruthenium glutathione sulfinate complexes).[86] Similarly, tandem MS approaches, CID and ETD, combined with IM-MS have been applied for the rapid determination of the binding site of the same Ru complex towards the undecapeptide and neurotransmitter substance P and in comparison with cisplatin.[78] ETD is a powerful radical-driven fragmentation method complementary to CID, useful for determining the sites of labile modifications of macromolecules. Combined with IM-MS, ETD provided both structural and conformational information on the adducts. Importantly, the MS experiments have shown a relatively weaker binding of the Ru complex compared with cisplatin towards the peptide. Furthermore, a comparison of CID and ETD demonstrated their complementarity: while the CID fragmentation technique was unable to detect any bound Ru species due to its high energy of fragmentation, ETD allowed to localise methionine as the favourite binding site for Ru.[78]

The potential interaction of Ru(II),[87-90] Ru(III)[90, 91] and dinuclear Ru(II)[92] anticancer agents towards oligonucleotides was also studied by ESI-MS. For instance, RAPTA derivatives as well as their osmium analogues were investigated for their reactivity towards a 14-mer single stranded oligonucleotide.[89] The results showed that while RAPTA-C gives rise to Ru-oligonucleotide mono-adducts following detachment of the arene, the osmium analogue forms both mono- and bis-adducts with maintenance of the arene moiety. As expected, [Ru(II)Cl$_2$(pta)$_4$] complexes were found significantly more reactive than the corresponding [Ru(III)Cl$_4$(pta)$_2$]$^+$ compounds towards a single strand oligonucleotide by ESI-MS.[90] Interestingly, the *trans*-Ru(II) complexes formed adducts of the kind [Ru(pta)$_2$]$^{2+}$ whereas the *cis* isomers exhibited a higher reactivity and gave rise to [Ru(pta)$_3$]$^{2+}$ adducts.[90] Similarly, ESI-MS has been used to analyse the reactivity of [(η^6-bipyridyl)Ru(en)Cl]$^+$ towards a 14-mer single stranded oligonucleotide. Mono- and di-ruthenated adducts have been described as the main products even in presence of potential competitive biomolecules such as histidine or cytochrome c, confirming the affinity of this Ru complex for DNA.[58]

A comparative study has been carried out by Groessl *et al.*, describing ESI-ToF and MALDI-MS as techniques for the analysis of adducts formed between duplex DNA and metallodrugs (Pt- and Ru-based), as well as the use of a top-down approach (CID-ESI-MS) to elucidate the binding sites.[73] In all cases, guanosine appeared as the preferential binding site for Pt(II), Ru(III) and even Ru(II) metallodrugs, with the following trend of adduct formation: cisplatin > oxaliplatin > NAMI-A > RAPTA-T > carboplatin > KP1019.

Notably, these results are in a good agreement with data obtained *via* other characterization methods (NMR, X-Ray diffraction) but whose require a significantly larger amount of compound, highlighting one of the main advantages of the use of mass spectrometry techniques. Finally, Sadler *et al.* reported on the comparison of the bottom-up and top-down approaches for analysing the competitive binding sites of a [(η^6-biphenyl) Ru(diamine)Cl]$^+$ complex on 15-mer single-strand oligodeoxynucleotides.[93] The bottom-up approach (ESI-MS combined with enzymatic digestion) allowed to localise the thermodynamically stable and favoured ruthenation sites on guanine residues, but was ineffective to determine less stable adducts that dissociate during digestion (with thymine residues for instance). In parallel, top-down experiments using CID fragmentation allowed the determination of thymine ruthenation sites, although induced the fragmentation of the G bases from the backbone.

2.3 Mass Spectrometry analyses of model proteins

In order to evaluate the molecular interactions of metallodrugs with proteins by MS in conditions reflecting the physiological ones, "soft" ionization techniques are required to ensure the conservation of the interactions and adducts formed. To this aim, MALDI-MS is not the most appropriate technique as interactions with the matrix can induce disruption of the metal-protein adducts, as well as artefacts in the resulting mass spectra. Furthermore, the MALDI technique suffers from a lack of sensitivity compared with ESI-MS, thus not giving a satisfying level of structural information. In the early 2000s, the first ESI-MS studies of the interactions between cisplatin and the model protein ubiquitin have been described by Gibson *et al.*.[75, 94-96] These pioneering experiments allowed the determination of possible competitive binding of platinum(II) drugs to proteins in the presence of DNA nucleobases. Besides ubiquitin, other proteins such as cytochrome c, calmodulin, insulin and lysozyme have been used as models over the last years to characterise the nature of metallodrugs' adducts. The main advantages of using model proteins with respect to protein targets, are that their sequence and structure are known, they have suitable molecular weights and are easily ionized. Below, we discuss the studies on the mostly investigated families of anticancer metal compounds with such model systems.

Ubiquitin (Ub)

One of the main model proteins used is ubiquitin (Ub), commercially available in high purity, with a reasonably low molecular weight (8.6 kDa). This protein is formed by 76 amino acids but only a few of them

(N-terminal Met1, His68, and a number of O donors) are available to become potential binding partners of metallodrugs. Furthermore, this protein doesn't contain redox active sites or disulfide bridges, simplifying the preparation processes.[37] Thus, in early studies, Gibson *et al.* described the use of ESI-MS approaches to compare the interactions of cisplatin and transplatin with ubiquitin.[75, 94, 95] From these experiments, clear differences in the nature of the adducts were highlighted among the two compounds. While cisplatin is forming predominantly bidentate adducts with ubiquitin (Ub-$Pt(NH_3)_2$), alongside with monodentate (Ub-$Pt(NH_3)_2Cl$ and (Ub-$Pt(NH_3)_2(H_2O)$) and tridentate species (Ub-$Pt(NH_3)$), transplatin is rather forming only monofunctional adducts (Ub-$Pt(NH_3)_2Cl$).[95] Experiments using ubiquitin with a chemical modification of the sulfur-containing methionine residue Met1, showed no interaction with cisplatin, thus leading to the conclusion that Met1 was the favoured binding site for cisplatin. More recently, other experiments were performed to characterise the interactions between platinum drugs and ubiquitin, using nanospray nESI-MS and MALDI-MS.[97] Upon incubation of the protein with cisplatin, transplatin and oxliplatin in a 2:1 protein to metal complex ratio, authors proved that in every case, only mono-adducts were detected, but of different nature. In accordance with the ESI-MS study, cisplatin formed mainly bidentate Ub–[$Pt(NH_3)_2$] adducts, while with transplatin the most abundant adduct corresponded to a monodentate Ub–[$Pt(NH_3)_2Cl$] species. Oxaliplatin formed exclusively bifunctional species of the type Ub–[Pt(DACH)]. Both ionization methods (nESI-MS and MALDI) lead to the same conclusions, although while nESI-QToF-MS allowed the unambiguous characterization of the adducts, MALDI-MS induced a higher degree of fragmentation. The nESI-ion trap IT-MS technique showed the advantage of higher sensitivity than the ToF instruments, allowing the detection of bis-adducts of oxaliplatin even after one week of incubation.

Bottom-up approaches have also been explored to gain structural information on the binding of cisplatin to ubiquitin. However, this protein possesses a very tight globular conformation and thus requires quite harsh conditions to be digested, leading to a highly probable breakage of the cisplatin-protein adducts.[94] Therefore, top-down mass spectrometry approaches have been described by Hartinger *et al.* to determine the precise binding sites of platinum-based drugs with Ub at a molecular level.[98, 99] In this study, high resolution FT-ICR and ESI-MS experiments using different MS^2 techniques (CID, IRMPD, HCD (high energy C-trap dissociation) and ETD) have identified the N-terminal methionine residue as the preferred binding site of both cisplatin and oxaliplatin. ETD revealed itself as leading to the larger extent of identified metal-peptides adducts and as allowing simultaneous and precise identification of Met1 and His68 as the preferential binding sites of oxaliplatin. Ion-mobility mass spectrometry was also applied to detect the

conformational changes induced upon platination of ubiquitin, and revealed the formation of up to three different conformations, all reduced in size compared to the starting free ubiquitin.[78, 100] The 3D conformation of a protein is of main importance for its biological activity, and in the case of ubiquitin incubated with cisplatin, up to three different conformations can be detected by IM-MS depending of the charge state. The collision cross-sections of each of those conformations indicated in every case a contraction of the platinated protein (on the N-terminal methionine) compared with native ubiquitin. Similarly, ion mobility combined with a top-down mass spectrometry approach has been used to characterise the reaction of platinum dicarboxylate complexes with ubiquitin. While targeted top-down MS experiments described the presence of bis- and tris-adducts and identified the N-terminal Met1 and C-terminal His68 as the preferential binding sites, ion-mobility technique revealed that the interactions of the platinum complexes with the protein resulted in a distribution of folded population of conformers (higher mobility, shorter drift times and small cross-sections).

ESI-MS and tandem MS experiments have also been used to study the interactions between gold- and ruthenium-based anticancer drugs with ubiquitin. Casini, Hartinger *et al.* reported on the use of FT-ICR-MS, ESI-MS, high resolution LTQ-Orbitrap, QToF and IT ESI-MS to characterise the interactions between ubiquitin and organometallic RAPTA-like complexes.[83, 87, 101-104] Only mono-, bis-adducts and in some cases tris-adducts have been detected, even in the presence of an excess of RAPTA complex, with the Ru(II) centre still bearing the arene ligand and in some case also the pta. In general, the studied organometallic ruthenium complexes exhibited a significantly higher reactivity towards the protein than the platinum drugs. The stability of the protein adducts towards biological nucleophiles has also been studied by ESI and FT-ICR-MS.[83, 103] Sulfur-containing biomolecules such as glutathione, cysteine and methionine could cleave Ub-Ru adducts, generating unbound protein; however, no detectable adducts with the nucleophiles could be detected.

In this context, Moreno *et al.* described the use of MALDI-ToF and IM-MS^2 approaches to study the influence of the ligands of organometallic ruthenium complexes towards their ability to interact with ubiquitin and more generally their anticancer activity.[105] The results obtained from both techniques revealed that the ruthenium compounds can interact with ubiquitin only if they do not possess PPh_3 as ligand, that seems to affect the adduct formation process and kinetics.

Concerning gold-based anticancer agents, only a few examples report on the use of mass spectrometry techniques to study their interactions with ubiquitin.[63, 106] As an example, Casini *et al.* used

ESI-QToF-MS to compare the reactivity of several gold(III) complexes towards this model protein. While the cyclometallated $[(N,N,C)Au(OH)]^{+}$ complex showed no reactivity towards Ub, the neutral organometallic $[(C,N)AuCl_2]$ compounds formed mono- and bis-adducts of general formula $[Ub+nLAu^{III}]^{+}$ (n = 1, 2), and the coordination complexes $[(N,N)AuCl_2]^{+}$ and $[(N,N,N)AuCl]^{2+}$ adducts showing only the presence of "naked" Au^{I} or $Au^{I}Cl$ fragments, with up to 3 gold atoms per protein.[65]

Cytochrome c (Cyt c)

Apart from ubiquitin, another widely used model protein to study the interactions with metallodrugs is the redox active cytochrome c, a small heme protein (12.000 Da, 104 amino acids) that is an essential component of the electron transport chain in mitochondria. This protein contains a single heme group covalently bound to the peptide via Cys14 and Cys17. Compared with ubiquitin, cytochrome c contains twice the number of methionine residues, 3 additional histidines and 2 cysteines; however, Met1 and His68 residues of Ub are more accessible than the above mentioned binding sites of Cyt c.

It is worth noting that, although cisplatin has been described as playing a potential role in the release of Cyt c from mitochondria to the cytoplasm,[107-109] this protein has been mainly used as model system in MS experiments. [104, 110, 111] Interestingly, while previous studies using different investigational methods, including MS, showed that cisplatin, transplatin, oxaliplatin and carboplatin possess different stabilities and reactivity with protein/peptides in physiological media, MS revealed that the compounds' behaviour towards Cyt c is quite similar. In most cases, the main adduct detected on the MS spectra corresponds to a 1:1 Pt/protein adduct. In the case of cisplatin and oxaliplatin, bis-adducts have also been detected as for ubiquitin, whereas transplatin can only form mono-adducts.[101, 110]

A determination of the binding sites of cisplatin towards Cyt c has been conducted using a bottom-up approach.[112] The primary adduct obtained by reaction of cisplatin and Cyt c, Cyt c-$Pt(NH_3)_2(H_2O)$, was digested by trypsin and its degradation followed by FT-MS, which revealed the presence of four new fragments that were analysed by tandem MS^2 and MS^3. Specifically, two fragments were identified as mono-adducts containing the $[Pt(NH_3)_2(H_2O)]^{2+}$ moiety bound to peptides (Gly56-Glu104 and Asn54-Glu104). The two other fragments were determined to be $[Pt(NH_3)_2(H_2O)]^{2+}$ species bound to heme-containing peptides (Acety1-Gly1-Lys53 and Acety1-Gly1-Lys55). The product ion spectra of these fragments allow the determination of the residue Met65 as the primary binding site of cisplatin to Cyt c. A combination of in-gel and in-solution digestion methods have also been described and allowed to determine multiple binding sites

for cisplatin.[113] Apart from Met65, the residues Met80 and Glu61/Glu62/Thr63 were found to react with cisplatin by liquid chromatography coupled with LTQ-MS. Alongside, to obtain information on the nature of the adducts and the mapping of the binding sites, FT-ICR MS has been used to observe the conformational changes of Cyt c induced by cisplatin.[114] The binding sites of cisplatin in Cyt c were confirmed after trypsin digestion to be Met65 (primary), Met80 (secondary), His18 and His33. Furthermore, in combination with hydrogen/deuterium exchange analysis, the platination of Cyt c by cisplatin at Met65 has been shown to disturb the protein's conformation leading to partial unfolding.

An interesting case study is the one related to the investigation of carboplatin with model proteins. Carboplatin, today among the most important platinum(II) anticancer drugs, manifests an extreme kinetic inertness *in vitro* at physiological pH and the actual mechanisms for its activation inside cells are still poorly understood. In this case, no significant reactivity was detected between carboplatin and proteins such as haemoglobin, ubiquitin as well as Zn_7-metallothionein by MS.[115-120] However, other studies by Casini *et al.* showed that Cyt c reacts with the drug, leading to the formation of stable platinum/protein adducts characterised by ESI-MS.[121] Specifically, binding of carboplatin to Cyt c can occur either through loss of the cbdca ligand or through a ''ring-opening'' reaction with retention of the cbdca (1,1-cyclobutanedicarboxylate) ligand and eventual release of ammonia. Further hydrolysis of the cbdca ligand yields *cisplatin-like* species. Notably, these adducts can react with guanosine 5'-monophosphate (5'-GMP), giving rise to the respective Cyt c–carboplatin–5'-GMP ternary complexes. Additional ESI-MS measurements on enzymatically cleaved Cyt c adducts suggest that protein platination probably occurs at Met65 as for cisplatin.

The search for new anticancer platinum drugs with improved pharmacological properties over those of cisplatin has been focused on *cis* geometric compounds for a long time. This bias originates from the fact that transplatin, the *trans* isomer of cisplatin, is not an active anticancer agent. However, since the first reports on *trans*-Pt complexes with relevant *in vitro* cytotoxicity, and in some cases endowed with significant *in vivo* activity,[122-125] *trans*-platinum complexes have been widely investigated as "rule breaker" anticancer drugs. In this context, the ESI-MS approach was extended to monitor the reactions of some cytotoxic platinum(II) iminoether complexes, *trans*- and *cis*-$[PtCl_2\{(E,E)\text{-}HN{=}C(OCH_3)CH_3\}_2]$ and *trans*- and *cis*-$[PtCl_2(NH_3)$[126]], with Cyt c (Figure 6A).[127] The investigation was independently supported by NMR, ICP-OES (inductively coupled plasma optical emission spectroscopy) and absorption electronic spectroscopy. From this study, it emerged very clearly that interactions with Cyt c do markedly alter the

intrinsic reactivity of the various platinum iminoethers, leading to the observation of rather unexpected chemical reactions at the level of the platinum ligands. In addition, the kinetics of degradation of the platinum complexes could be measured and found to be largely affected by the interactions with this protein. Remarkably, a profoundly different pattern of reactivity was identified for the *trans* isomers with respect to the *cis* ones (Figure 6B).

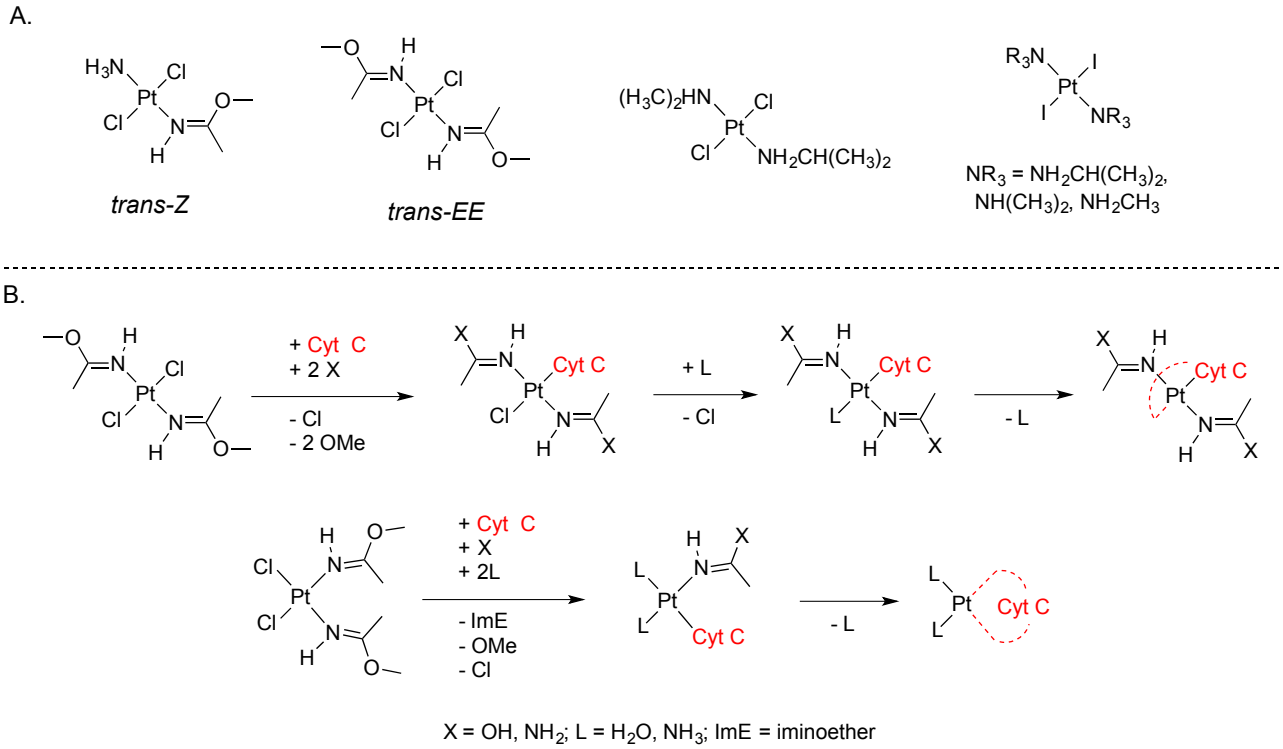


Figure 6 - A. Scheme of some *trans*-platinum complexes studied for their interaction with Cyt c.[127-129] B. Proposed reaction scheme for complexes *cis*- and *trans-EE* with Cyt c, adapted from Ref [127].

Among the cytotoxic *trans*-Pt(II) complexes, those with aliphatic amines as ligands (non-leaving groups), of general formula *trans*-[PtLL'Cl_2] (L and L'=aliphatic amines) hold promise. The results obtained through ESI-MS provided evidence that Cyt c undergoes appreciable, although sub-stoichiometric,

platination, upon reacting with two complexes of this family.[128] This was confirmed by ICP-OES measurements. Both mono- and bis-platinum adducts with Cyt c were identified and the main molecular fragment which is eventually bound to Cyt c contains the platinum centre, as well as the two ammine ligands. The investigation was extended to *cis/trans*-[PtLL'I_2] complexes, with iodido ligands, and screened their reactivity with sulfur donors of thioether or thiol type, such as N-AcMet and N-AcCys, respectively, and with Cyt c.[129] Interestingly, these studies revealed a very unusual reactivity profile for the three *cis*-type platinum complexes featuring the release of the ammine ligand with partial or complete retention of the iodido ligands upon binding to model amino acids or peptides. In contrast, *trans*-type complexes manifested a lower propensity to form adducts with amino acids or with Cyt c, and a more classical reactivity consisting of the preferential release of the iodido ligands upon protein binding was revealed.

Alongside cisplatin and other platinum-based complexes, experimental anticancer ruthenium and gold compounds have been studied by MS techniques for their affinity towards Cyt c. For example, ESI and LTQ-Orbitrap MS techniques have been used to characterise the adducts between antimetastatic NAMI-A and RAPTA-like complexes with this model protein.[87, 110, 130, 131] In the case of RAPTA compounds, both approaches described the presence of mono-adducts of the type Ru(η^6-*p*-cymene) and to a lesser extent Ru(η^6-*p*-cymene)(pta). ESI-MS spectra recorded over time allowed the study of the kinetics of formation of each adduct.[110] After 3 h incubation, the peaks associated with both mono-adducts were observed in a very limited extent. After 6 h, the signals corresponding to the adducts increased, mainly the Ru(η^6-*p*-cymene). Finally, after 12 h, the main peak observed on the MS spectrum (60% relative intensity) corresponds to the Cyt c-Ru(η^6-*p*-cymene) adduct. Similar adducts were detected by ESI-MS analysis of Cyt c incubated with 10-fold excess of the cytotoxic organometallic [(η^6-arene)Ru(en)Cl]$^+$ complex, revealing the formation of the main adduct Cyt c-(η^6-arene)Ru(en)$^+$. Interestingly, lowering the pH from 7.4 to 7.0 resulted in significant reduction of adduct formation, until no ruthenation was observed at pH 5.5.[58]

Similarly, ESI-MS experiments were reported to study the interactions between the coordination Ru(III) complex NAMI-A and Cyt c, revealing a high reactivity.[131] At mixing, apart from the native protein, a peak corresponding to an adduct with a non-covalent [$RuCl_4$(DMSO)(Im)]$^-$ fragment was detected, and after 2 h incubation, a group of peaks at lower masses appeared, indicating that ligand exchange reactions occurred to favour direct coordinative binding of the ruthenium centre to Cyt c. After 24 h, a single peak was observed, corresponding to an adduct of the protein with a hydrated Ru ion, indicating its loss of every original ligands.[131]

Casini *et al.* used LTQ-Orbitrap ESI-MS to analyse the reactions of metal compounds with mixtures of selected proteins. Specifically, three medicinally relevant representative compounds, cisplatin, transplatin and the organometallic ruthenium compound RAPTA-C (Figure 1), were reacted with a pool of three proteins, namely ubiquitin, cytochrome c and superoxide dismutase (SOD), and the reaction products were analysed using high-resolution mass spectrometry.[101] The formation of metal–protein adducts was clearly observed upon incubation with the compounds. In addition, valuable information was obtained on the nature of the protein bound metallo-fragments, on their distribution among the three different proteins and on the binding kinetics. The platinum compounds were less reactive and considerably less selective in protein binding than RAPTA-C, which showed a high affinity towards Ub and Cyt c, but not SOD. In addition, competition studies between cisplatin and RAPTA-C showed that the two metallodrugs have affinities for the same amino acid residues on protein binding.

Such information has important implications on the mode of action of the metallodrugs in cells and presumably also on their possible toxic side effects. This latter type of MS experiment should be further developed in future target identification studies. Once actual protein targets have been established, it would be interesting to screen them in the presence of other proteins, as described here, to provide an indication of the compounds' selectivity.

Concerning cytotoxic coordination gold(III) complexes, a number of derivatives - mononuclear (N^N)Au(III) and dinuclear Au(III)-*μ*-oxo dinuclear complexes - were reacted with Cyt c and their adducts studied by ESI-MS. Initial experiments revealed a fast reduction of the complexes to form only Au(I) adducts with release of the bidentate N^N ligands.[104, 132, 133] Independently on the nature of Au(III) coordination complexes, a similar reactivity towards Cyt c was noticed, consisting in the reduction of Au(III) to Au(I) upon protein binding, and concomitant release of the ligands. However, the above mentioned ESI and ESI-QToF MS experiments performed with cyclometalated Au(III) complexes of general formula $[(N^N^C)AuOH]^+$ and $[(C^N)AuCl_2]$ revealed the presence of small amount of adducts corresponding to Cyt c-$[n(L+H)Au(III)]^+$ (n = 1, 2), most likely due to the higher redox stability of Au(III) ions within cyclometalated scaffolds in comparison with coordination N^N ligands.[65, 133, 134] This finding was further confirmed by comparing the reactivity towards cytochrome c of 2-substituted pyridine-based coordination cationic $(N^N)AuCl_2^+$ as well as neutral $(N^N)AuCl_2$ and (N^N^O)AuCl complexes using ESI-MS.[135] In this case, while all complexes exhibited high reactivity towards Cyt c, the cationic compound formed only adducts of the type Cyt c+nAu^I (n = 0-4)

with reduction of the gold centre and complete loss of the original ligands, whereas the neutral compounds were shown to form adducts of the type Cyt c+n[$Au^{III}(L)]^+$ (n = 0-4).

In the case of the gold(I) anti-arthritis agent auranofin, ESI-MS spectra revealed the presence of adducts corresponding to the intact complex bound to Cyt c, probably *via* non-covalent interactions, and of Cyt c+$Au(PEt_3)$ mono-adducts, as already reported with another model protein (lysozyme).[133, 136]

Hen Egg White Lysozyme (HEWL)

Ubiquitin and cytochrome c represent the two most frequently used model proteins to study the interactions with metallodrugs by mass spectrometry techniques, but other proteins have also emerged in the last decade. Among them, hen egg white lysozyme (HEWL) has been described and consists in a relatively small protein formed of 129 amino acids. The rather specific aspect of this enzyme of biological significance relies on its stable tri-dimensional structure associated with the presence of four disulfide bonds between cysteine residues alongside the peptide chain.

ESI-MS experiments have been optimized to study the interactions of cisplatin and other platinum-based anticancer drugs towards this protein.[137] The first observation drawn from this study is the significantly slower interaction between the complexes and HEWL in comparison with both Cyt c and Ub. Even after 72 h incubation, the peak corresponding to the native protein was found to be the highest of all others. The interactions of cisplatin and its analogues, transplatin, carboplatin and oxaliplatin, with HEWL were analysed by ESI-MS, and the resulting metallodrug–protein adducts identified. All platinum complexes were shown to be less reactive with HEWL than with Cyt c and Ub, forming mainly mono-adducts. Specifically, the ESI-MS spectrum of cisplatin showed adducts corresponding to $[Pt(NH_3)_2Cl]^+$ or intact cisplatin bound to the native protein, in accordance to the results obtained with the other model systems, and in a lesser extent some adducts corresponding to doubly platinated species.[138] The carboplatin mass spectrum revealed the presence of an adduct corresponding to the addition of a $[Pt(NH_3)_2$cbdc] fragment (cbdc = cis-(1,1-cyclobutanedicarboxylate) to the protein, as a result of the classical ring opening process, while reaction of oxaliplatin gave rise to a $[Pt(DACH)]^{2+}$ fragment bound to HEWL (with concomitant release of the oxalate ligand), as found for Cyt c.[110] Finally, the same experiment performed with transplatin or *trans*-Pt(II) derivatives evidenced the binding of a $[Pt(NH_3)_2Cl]^+$ fragment to HEWL with release of a single chlorido ligand.[137] In the same study, the X-ray crystal structure of the cisplatin lysozyme derivative revealed selective platination of the Nε of the imidazole ring of His15 residue.[137] It is very likely that this

residue represents a general binding site for platinum drugs and other late transition metal complexes on HEWL.

Recently, a bottom-up mass spectrometry approach has been reported to identify and confirm the binding sites of cisplatin to HEWL.[139] Due to its highly stable 3D structure associated with the presence of multiple disulfide bonds, the digestion of HEWL prior to analysis proved to be challenging, initially requiring a pre-reduction of the disulfide bonds prior to trypsin digestion. However, such treatment could easily reverse the binding of platinum drugs, so several digestion methods have been envisaged to optimise the analysis of the platinated binding sites: trypsin digestion, DTT reduction followed by trypsin digestion, digestion at high temperature (55°C) and finally digestion in presence of an organic solvent (acetonitrile). The fragments obtained were then analysed by FT-ICR MS and compared. The simple trypsin digestion appeared to be inefficient, and the reduction of disulfide bonds by DTT or TCEP destroyed the platinum binding sites, probably because of the high affinity of sulfur (in DTT) and phosphorus (in TCEP) for platinum. A combination of trypsin and heat at 55°C revealed itself to be an efficient method of digestion in a few hours, as well as addition of 60% acetonitrile. Both digestion methods allowed the detection by MS of platinated peptides fragments, which were then analysed using SORI (Sustained Off-Resonance Irradiation) CID (CID technique used in FT-ICR MS involving the acceleration of ions in the cyclotron motion and the increase of the pressure resulting in the production of CID fragments) to identify the binding sites of platinum. Importantly, while X-ray diffraction analyses allowed the determination and identification of only one binding site for cisplatin to HEWL, the His15 residue,[137] the results using the bottom-up approach and MS^2 showed several targets sites. In the peptide 15-21, His15 was found to be the primary binding site of cisplatin, as detected by X-Ray diffraction, while Glu35 and Ser36 were identified as potential binding sites for platinum residues in the peptide 34-45 [137]. Similarly, in the fragment 46-61, platinum ions were found to bind to Asn-Thr-Asp (46-48) without unambiguous identification of the precise atomic site, threonine and aspartic acid having both a potential affinity for platinum. Finally, Ser100 and Asp101 were observed to be both potential targets of platinum for the fragment 98-112.

Other cisplatin-related complexes have been evaluated for their affinity towards HEWL using LTQ-Orbitrap ESI-MS. Depending on the nature of the ligands, different platinum adducts were detected. For example, bidentate S^O ligands were shown to remain bound to Pt(II) upon protein adduct formation, whereas N^N ligands were released upon protein binding.[140, 141]

A few examples of ruthenium-based anticancer complexes have been described for their reactivity towards HEWL using MS techniques. Using LTQ ion trap and Orbitrap ESI-MS, NAMI-A was studied for its affinity to the protein.[131] In each reported condition, the main peak detected on the spectra was identified as the native protein, indicating a low reactivity of the complex for HEWL. The main adducts detected were found to be $[HEWL+RuCl_4(DMSO)(Im)]^-$ (non-covalent binding) and $HEWL+RuCl_3(DMSO)(Im)$ (potentially *via* covalent binding). Non water-soluble Ru complexes of the general formula $[(\eta^6\text{-p-cymene})RuCl(N\text{^}N)]^+$ were similarly investigated and the only adducts detected were found to be $[HEWL+Ru(\eta^6\text{-p-cymene})]$ and $[HEWL+Ru(\eta^6\text{-p-cymene})Cl]$ with complete detachment of the bidentate N^N ligand.[140]

Both Au(I) and Au(III) complexes have also been studied for their reactivity towards HEWL using LTQ-Orbitrap ESI-MS.[142] The nature of the adducts were found to be quite similar to the ones obtained with Cyt c. N^N coordination Au(III) and dinuclear Au(III)-*μ*-oxo dinuclear complexes have been described as forming adducts of maximum four naked Au(I) to the protein, potentially on the Met65, Met80, His18 and His33 residues as previously reported.[140, 143] A cyclometalated $(N\text{^}N\text{^}C)Au(III)^+$ complex has proven its ability to form adducts preserving the oxidation state of Au and its N^N^C ligand upon binding to HEWL. On the other hand, auranofin was studied in the same conditions and the main adduct detected upon reaction with HEWL was the intact complex, probably *via* non-covalent interactions, alongside a lower intensity signal corresponding to the mono-adduct $[HEWL+Au(PEt_3)]^+$, as described for Cyt c.[136, 142]

The presence of reducing agents such as glutathione and ascorbic acid was studied to evaluate the influence on the nature and kinetics of adducts formation. It was determined that ascorbic acid was able to induce formation of higher amounts of metal-protein adducts with the Au(III) coordination complexes, by accelerating the reduction process from Au(III) to Au(I), whereas glutathione tended to reduce the formation of adducts by sequestering the Au(I) ions.[142]

Calmodulin

Calmodulin (Calcium-Modulated protein) is a small and highly conserved protein of 148 amino acids that offers 4 Ca^{2+} ions binding sites. Calmodulin contains a large amount of residues that represent potential binding sites for metallodrugs (9 Met, 1 His, 17 Asp and 23 Glu) and thus represent a great challenge to identify cisplatin's binding sites. Sadler *et al.* have reported the use of a variety of MS and MS^n techniques in both top-down and bottom-up approaches to map the platination sites on calmodulin.[80, 144, 145] Cisplatin was identified as forming mono-adducts on the protein as well as cross-links in between two peptides

fragments (107-126 and 127-148). Tandem MS experiments have allowed the identification of the precise binding sites as being the C-terminal Met144 or Met145 and the Met109 residues.[144] In presence of a large excess of cisplatin, other binding sites were identified as mainly other Met residues.

Insulin

Insulin is a hormone protein that regulates the metabolism of carbohydrates and fats. The human protein contains 51 amino acids that are organized as a dimer of 2 chains (A, 21 amino acids, and B, 30 amino acids) linked together *via* disulfide bonds between cysteine residues. Top-down and bottom-up MS approaches have been reported to describe the interaction of cisplatin and oxaliplatin towards insulin.[79, 146-149] ESI-MS experiments confirmed the ability of insulin to react with cisplatin, forming mainly bidentate adducts of the type [insulin+$Pt(NH_3)_2$]$^{2+}$ or monodentate as [insulin+$Pt(NH_3)_2Cl$]$^{+}$ depending on the pH and the experimental parameters used, with up to 3 bound Pt(II) ions.[146, 147] Gomez-Gomez *et al.* reported on the comparison of top-down and bottom-up approaches to localize the binding sites of platinum.[146] CID-MS^n experiments have led to the identification of B-chain N-terminus His5, His10 and in a lesser extent Cys7 residues as the preferred binding sites. It is worth mentioning that digestion of the platinated protein led to the conservation of insulin-platinum adducts. Direct analysis of these fragments has allowed the determination of a larger amount of potential platinum binding sites (B-chain N-terminus His5, His10, Cys7, Cys19 and A-chain Cys6, Cys7 and Cys20), indicating that, in this case, a bottom-up approach is more appropriate. A top-down approach using ECD fragmentation has nevertheless been described to evaluate the potential of cisplatin as a cross-linking agent for insulin.[79] The results concluded that $[Pt(NH_3)_2]^{2+}$ fragment was able to form an adduct between the B-chain Lys29 residue of an insulin chain with a B-chain Glu21 residue of another insulin chain. Similarly, oxaliplatin has been described as reacting with the His5 residue of a B-chain of insulin.[148] Reduction of the disulfide bonds has allowed the additional identification of two binding sites on the A-chain Cys6 and B-chain His5 residues, while a bottom-up approach confirmed the determination of the binding sites with the top-down approach plus the identification of a supplementary Cys7 residue. Interestingly, Sadler *et al.* applied the same top-down approach to compare the interaction of a [(η^6-biphenyl)(bipyridyl)RuCl]$^{+}$ complex with insulin.[84] In this case, the preferred binding sites were identified as His10, His5 and in a lesser extent Glu13 and Tyr15 (potentially *via* π-interactions).

2.4 Competition experiments with GSH

Several reports concern the study of possible reversible binding of metallodrugs to their targets, fostered by other biological nucleophiles by MS. As previously mentioned, glutathione (GSH) is a sulfur-containing peptide that can function as a reducing agent and as a detoxification agent towards metallodrugs. Therefore, a number of studies investigated the stability of metallodrugs-protein adducts in presence of GSH by MS. Gibson *et al.* first reported competitive ESI-MS experiments of ubiquitin-Pt adducts in presence of GSH.[75] Peaks in the mass spectra were attributed to $Pt(NH_3)(Ub)(GSH)$ adducts, where GSH is most likely bound to Pt(II) in a monodentate fashion. Similarly, the stability of the adducts formed between oxaliplatin and 5'-GMP in presence of GSH has been evaluated using HPLC coupled to ESI-ToF-MS.[150] The results indicated that GSH could produce a number of conjugates, interfering with the initially formed Pt-nucleobase adducts (Figure 7, species I and II) in two different ways: *i)* by binding to Pt(II) in concomitance to the nucleobase as in the case of cisplatin (Figure 7, species III and IV), and/or *ii)* by substituting the 5'-GMP ligand(s) (Figure 7, specie V), respectively.

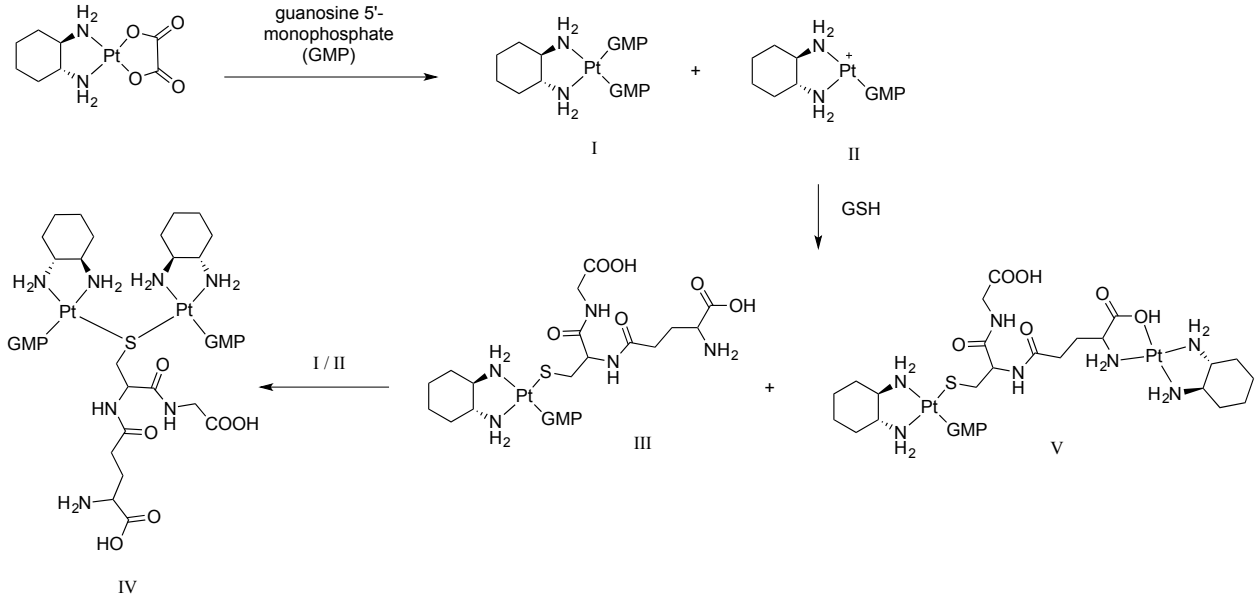


Figure 7. Proposed reaction pathway of the reaction between oxaliplatin and 5'-GMP in presence of GSH. Adapted from Ref [150].

Sadler *et al.* evaluated the competitive reaction of a $[(\eta^6\text{-biphenyl})(en)RuCl]^+$ towards GSH and single/double stranded oligonucleotides.[85] The ruthenium complex was characterised as forming very stable adducts with the single stranded oligonucleotides. In the case of double stranded oligonucleotides, both thiolato and sulfenato intermediate and unstable adducts were detected by MS. Globally, the study concluded that the ruthenium complex most likely underwent hydrolysis, coordination of a single molecule of GSH, oxidation, and finally formation of stable mono-adducts on guanosine residues of the strands.[85]

Furthermore, the stability of RAPTA-C-Ub adducts in presence of GSH has been evaluated by FT-ICR-MS experiments.[83] Upon addition of GSH, the FT-ICR-ESI-MS spectrum did not change significantly in comparison with the original sample. In fact, after 24 h incubation, the only noticeable difference was found to be the increase of the peak corresponding to the free Ub, released from the Ub-Ru adducts, while no adducts were detected containing GSH fragments. Such slow release mechanism was indicated as an important indication concerning the mode of action of RAPTA-C, suggesting that ubiquitin could serve as a reservoir that releases small amounts of cytotoxic Ru species over time.[83]

3. Interactions of metallodrugs with serum and serum proteins

Most metal-based drugs are nowadays administered to patients intravenously. Therefore, determining the interactions and the nature of the possible adducts formed during and after injection between the metallodrugs and the blood constituents is crucial. Serum, defined as the liquid fraction remaining after blood coagulation, is a highly complex biological fluid that contains thousands of different proteins at concentrations varying from mM to pM. Among the main proteins in the serum, albumin, transferrin and globulins may interact with metal ions/complexes. Indeed, serum proteins are thought to play a leading role in the transport, delivery, distribution and storage of metallodrugs. Moreover, most serum proteins possess several disulfide bonds that might complicate the network of possible interactions.

The most abundant human serum protein is albumin (HSA), found in blood at a concentration of around 600 μM, consisting of 585 amino acids. At physiological pH, the protein exhibits a helical conformation, with 17 disulfide bonds and 2 residues in hydrophobic cavities accessible for potential metal binding/interaction (Cys-34 and Trp-214).[151] Human serum transferrin (hTf) is found in blood at a concentration of around 35 μM, and is composed by 679 amino acids; its main role is to transport Fe(III) ions (up to two Fe(III) bound per mole). Each iron binding site in hTf exhibits a distorted octahedral geometry between two tyrosines, one histidine and one asparagine, with a bidentate carbonate ion acting as a synergistic anion in the process of Fe(III) binding.[151]

Overall, a comprehensive MS analysis of metallodrugs interactions with serum and its components presents a high degree of complexity. Such investigations require the use of one or several consecutive separation techniques (chromatographic, such as SEC, or electrophoretic, such as CZE) prior/coupled to analysis by ESI- or ICP-MS techniques.[37] SEC has been used widely for the analysis of serum proteins incubated with metallodrugs thanks to its compatibility with pseudo-physiological media, its sensitivity and its

ease of hyphenation to MS instruments such as ICP-MS. However, the CE technique coupled to MS techniques has also the advantage of low sample consumption and high separation ability, while maintaining a good compatibility with physiological conditions. This criterion is essential to avoid interferences, which might affect the nature, and/or the stability of the metal-protein adducts. Indeed, factors such as pH, organic solvents, buffer components, ionic strength etc. might influence the reactivity of the metallodrug towards the proteins of interest.

In this context, cisplatin has been widely studied for its interactions with serum proteins using a plethora of analytical techniques, and the main conclusion drawn has been that both transferrin and albumin are the preferential binding partner, along with other proteins that have crucial roles in the pharmacokinetics and delivery of the drug, for example acting as a reservoir.[151] Nevertheless, it is worth mentioning that there is still some conflicting information on the affinity of cisplatin for serum proteins, some reports claiming preference for transferrin,[151] others selectivity for albumin[33], as well as on the platination sites on those proteins.[152]

Interactions between cisplatin and albumin have been demonstrated to occur,[153] most likely *via* the high affinity of platinum for sulfur donors. Thus, Cys34 in HSA has been considered as the most likely binding site, also due to its accessibility, but other residues such as methionine or histidine may also offer additional binding sites.[151, 152, 154] As an example, Sheldrick *et al.* developed a shotgun proteomic approach called the multidimensional protein identification technology (MudPIT) to identify the binding site of cisplatin on serum proteins.[155] As previously mentioned in the introduction, MudPIT combines 2D SCX (strong cation exchange) and RP (reversed phase) chromatography with ESI-MS^2, and allows up to 1,500 proteins to be characterised in a 24 h period. *Via* this method, five binding sites were described for cisplatin on HSA, specifically Cys34, Met329, Met548, Tyr150 (or Tyr148) and Asp375 (or Glu376).[155] In the same study, Met256 was established as a cisplatin coordination site for hTf in addition to the O-donor sites Glu265, Tyr314, Glu385 and Thr457. Inspection of the protein structures indicated that the preferred residues belong either to peripheral helices or to flexible loops within the protein-binding pockets. Notably, O-donor residues dominate as cisplatin binding sites for other abundant serum proteins. It is worth mentioning that the cisplatin's coordination sites in HSA and hTf were confirmed by independent MudPIT studies on cisplatin reaction mixtures with the individual proteins.[155]

A complementary study by Gomez-Gomez *et al.* using a gel-based bottom-up approach by nHPLC-LTQ-ESI MS/MS proposed that Met329, His338, His288, Cys289 (although participating in a disulfide bond

in the native protein) and Met298 residues are the potential platination sites for cisplatin in HSA.[113] Cisplatin has also been found to cleave initial S-S bonds after coordinating Cys289 (and possible intramolecular Pt(II) coordination to two sulfur atoms), which could then induce distortion of protein conformation and loss of the protein's biological activity.[113, 151] A bottom-up approach (tryptic digestion followed by LC-MS/MS experiments) described by Sadler *et al.* also revealed the ability of cisplatin to cross-link two histidine residues at the interface between the two domains of HSA.[156] Notably, a shotgun approach including FASP (Fast Aided Sample Preparation) protein digestion, peptide-based OFFGEL-isoelectric focusing (IEF) fractionation (separation of peptides and proteins according to their isoelectric point using immobilized pH gradient gel strips) and nLC-ESI-MS/MS has been recently reported to investigate cisplatin's binding sites on HSA and revealed more potential platination sites (His9, His67, His105, His128, His247, Met298, Met329 and Asp13).[157]

In binding studies of cisplatin with purified serum transferrin, Dyson *et al.*[158, 159] reported on a single platination site,[113, 158, 159] using a bottom-up approach using a hybrid HPLC-MS^2 technique. In this study, Thr-457, involved in the Fe(III) binding site of the C-terminal lobe of the protein, has been determined as the preferred platination site, featuring a transferrin-*O*-Pt$(NH_3)_2$Cl adduct. Molecular modelling experiments confirmed the stoichiometry of the binding and suggested that cisplatin can occupy part of the iron-binding site in the C-terminal lobe, as well as the carbonate's site, thus preventing Fe(III) binding.[159] Interestingly, using a model peptide of the transferrin iron binding pocket, another ESI-MS study revealed the binding ability of cisplatin to a Cys residue, although it is worth mentioning that such residue is engaged in a disulfide bond in the native protein and may not be relevant in physiological conditions.[36] Of note, a gel-based bottom-up approach using nHPLC-ESI-LTQ MS/MS on purified transferrin rather concluded that the Met382 residue was the most favourable hTf platination site,[113] in agreement with previously reported NMR studies.[160]

Competitive experiments using HPLC-ICP-MS and reacting cisplatin with several plasma proteins determined a preference of the metallodrug for HSA after 24 h incubation (complete complexation versus 50% in the case of transferrin) and for haemoglobin.[33, 161] Haemoglobin (Hb), the iron-containing oxygen-transport metalloprotein found in red blood cells, has also been considered as potential target for cisplatin.[118] Using nESI-MS, platination was demonstrated to take place with loss of both chlorido ligands. A combination of size-exclusion HPLC and ICP-MS detection of both iron and platinum techniques

concluded that after 24 h, platinum was mainly bound to the intact haemoglobin without loss of the iron ion, while in certain adducts heme loss was noticed.[118]

Although all of the three Pt drugs can form complexes with Hb, the degree of complex formation varies. Experiments using both RBCs and Hb demonstrated that the ability of the three Pt drugs to bind with Hb follows the following decreasing order: oxaliplatin > cisplatin > carboplatin. This coincides with the order of their ability to form reactive species (one or two ligands are replaced by water). In fact, Hb-carboplatin adducts (one still containing the heme group, one without) were only detected after 4 days of experiment in a negligible amount.[118, 161] Interestingly, the formation of Hb−Pt complexes is accompanied by the corresponding release of Fe (heme group) from the Hb molecule, as confirmed by using size fractionation, size exclusion HPLC-ICP-MS, and nanospray MS. Generally, and at variance with oxaliplatin and cisplatin, carboplatin has scarce affinity for both HSA and haemoglobin.[161]

nESI-QToF-MS and HPLC-ICP-MS experiments concluded that oxaliplatin was forming adducts with transferrin as the intact parent molecule as well as its hydrolysed species, in a stoichiometry that was concentration-dependent, but with no information of the potential binding sites on the protein.[162]

The trinuclear platinum complex BBR3464 has also been characterised for its interactions with purified HSA using multiple techniques including ESI-MS.[163] Thanks to the central platinum moiety, a pre-association with target biomolecules by hydrogen-bonding or electrostatic interactions has been demonstrated in several examples.[164, 165] A similar trend was observed by ESI-MS upon incubation of BBR3464 with HSA, with rapid (few min) establishment of non-covalent interactions.[163] Interestingly, the possibility of non-covalent adduct formation between metallodrugs and proteins has also been reported in the case of the ruthenium(III) complex NAMI-A and the model protein lysozyme.[131]

Ruthenium-based complexes have also been studied for their interaction with serum proteins using different MS techniques. Once more, the results described in the literature concerning the selectivity and the affinity of ruthenium-based metallodrugs with serum proteins are quite controversial. In some reports, transferrin has appeared as a preferred binding site for the Ru(III) complex KP1019, with an average of two ruthenium centres per transferrin as determined by ESI-MS, and with a binding selectively on the Fe(III) site as confirmed by other techniques.[166] Albumin was also described as favourable target for KP1019 using different analytical methods, and the compound was found to bind to nitrogen atoms of histidine side chains.[167]

Using similar techniques, NAMI-A was found to bind serum albumin in a 2:1 ratio (Ru:protein).[126, 168] Using SEC-ICP-MS, after 24 h incubation, NAMI-A was found mostly (95%) bound to both transferrin and albumin, but with a faster and higher reactivity towards albumin. In any case, the ruthenium adducts were stable over time (several days).[126, 169, 170] While one-dimensional SEC-ICP-MS allowed the determination of the molecular sizes of the targeted proteins interacting with KP1019 and thus their identification, two-dimensional SEC-IC-ICP-MS (hyphenation of multi-dimensional SEC-IC chromatography to ICP-MS) gave quantitative information on the adducts.[171] The nature of the adducts and the influence of the presence of reducing agents at the average cancer cytosol concentration on the reactivity of KP1019 towards serum proteins was evaluated by CE-ICP-MS.[172] Interaction of KP1019 with holo-transferrin gave rise to adducts with no displacement of Fe(III) from both binding sites, suggesting a binding of ruthenium on other sites that those involved in Fe(III) binding.[172] Glutathione was able to entirely remove iron from transferrin in less than 3 h. Ruthenium release from the same protein was found to be significantly slower (complete removal in 24 h).

Further identification of ruthenium-transferrin adducts (by incubation of KP1019 with apo- and holo-transferrins) by ESI-ToF-MS and ESI-QqQ-MS (triple quadrupole analyser) experiments has been reported by Timerbaev *et al.*, in buffer and in presence of reducing agents (cancer cell cytosol conditions).[173] In ammonium chloride buffer (simulated extracellular conditions), KP1019 was found to interact with all isoforms of both apo- and holo-transferrins, forming mainly bis-adducts, with the ruthenium centre still in the oxidation state +III and conserving one or both the indazole ligands. In cancer cell cytosol conditions, the protein adducts were all identified as containing Ru(II) fragments, supporting the 'activation by reduction' hypothetical mode of action of Ru(III) anticancer agents.[173] The potential influence of the nature of the counter anion of KP1019 on the type of interactions with serum proteins has been evaluated by several MS techniques using the sodium salt of KP1019 (KP1339) and demonstrated the absence of any effect, showing the same reactivity for both Ru(III) complexes.[174, 175]

The experimental anticancer Ru(II) complex RAPTA-T [Ru(η^6-toluene)Cl_2(pta)] has also been studied for its reactivity towards serum protein using MS techniques.[36] Specifically, SEC-ICP-MS experiments were performed to study the interactions between the ruthenium complex and holo- and apo-transferrin and HSA. RAPTA-T binds equally to both holo- and apo-transferrin, and to a significantly greater extent compared with cisplatin. The ruthenium compound exhibited a kinetic preference for holo-hTf (up to 1.4 Ru per protein) compared with apo-hTf and HSA (0.5-1.0 Ru per protein). However, it is worth reminding

that HSA is 20 times more concentrated in human serum, and thus, HSA can still be considered as a favoured site for metal binding, as shown for KP1019.[171] The binding of ruthenium to the proteins has appeared as concentration-dependent: higher concentrations of both the metal complex and proteins promote a higher degree of interaction.[36] Both RAPTA-T and cisplatin had the same effect on the release of iron from the holo-transferrin: free Fe(III) was only detected upon incubation with a large excess of the metallodrugs. Identification of the nature of the adducts and the binding sites were achieved by ESI-MS experiments.[36] Upon binding to transferrin, loss of the two chlorido ligands, followed by detachment of pta, was observed for RAPTA-T, together with the coordination of a carbonate anion to the ruthenium centre. The maintainance of the arene ligand was already reported in the case of the Cyt c protein.[130] Afterwards, a model peptide containing the active site sequence (13 amino acids) of hTf was used to identify the Ru binding sites by tandem MS using CID fragmentation.[36] This peptide contains a specific His residue (His249) involved in Fe(III) binding in the native protein, and a number of other histidine and cysteine residues as additional potential nucleophiles. RAPTA-T formed tight adducts at one of the histidine residues that is not involved in iron binding in the native protein, with concomitant loss of the two chlorido ligands, thus confirming the inertness towards the Fe(III) binding site.[36]

Interestingly, experimental cytotoxic dinuclear ruthenium(II) arene-complexes - 1,2-bis{chlorido[3-(oxo-*K*O)-2-methyl-4(1H)-pyridinonato-*K*O4](*η*^{6}-*p*-cymene)ruthenium(II)}alkane - were evaluated for their reactivity towards transferrin using ESI-MS techniques.[92] Similarly to RAPTA-T, a fast reactivity towards the protein was highlighted, featuring adduct formation upon loss of the chlorido ligands, and with up to maximum two molecules of complex per protein, even in the presence of a large excess of the metal compound.

Finally, concerning gold complexes, the antiarthritic gold(I) complex auranofin has been scarcely characterised for its interactions with serum proteins as its administration is done orally. However, a few studies using various techniques have demonstrated the reactivity of Au(I) complexes towards HSA, which could act as a potential carrier.[176, 177] Thus, ESI-MS was also used to study the interactions between $[Au(S_2O_3)_2]^{3-}$ and HSA.[178] After 1 h incubation, adducts corresponding to 'naked' Au and the intact $[Au(S_2O_3)_2]^{3-}$ complex were detected, and the residue Cys34 was hypothesized as the most likely binding site, being the only free thiol in native HSA.[178] Recently, CE-ICP-MS methods were applied by Gammelgaard *et al.* to analyse the reactivity of auranofin towards HSA and human plasma.[179] The results showed that auranofin reacts quickly with the considered proteins, with more than 95% of protein-bound

auranofin detected after 1 h incubation. Pre-alkylation of the residue Cys34 induced a significantly decreased reactivity of the gold compound towards HSA, thus supporting the hypothesis of Cys34 being the main binding site for Au(I) complexes.[179] However, other reaction sites might have been affected by the modification of the protein, so it cannot be excluded that a small fraction of auranofin may be bound to other residues.

4. Interactions of metallodrugs with pharmacological targets

4.1 Copper transporters and chaperons

Copper represents an essential element for most eukaryotic organisms as it is a cofactor for many enzymes and is involved in several biological processes such as mitochondrial respiration, iron metabolism or free radical detoxification.[180] Intracellular Cu(II) ions are highly toxic ions as they can form free radicals by reaction with molecular oxygen or hydrogen peroxide. Therefore, an efficient copper regulation and homeostasis is necessary to guarantee their utilization by copper-dependent proteins or their elimination, assuring a protection of the cells from excess free copper at low oxidation state. In mammals, Cu^{+} enters inside cells *via* the protein copper transporter 1 (Ctr1). Once taken up, copper is then trafficked intracellularly by various chaperones such as the antioxidant protein 1 (Atox1) or the cytochrome c oxidase assembly homolog (COX-17) *via* several possible pathways, and is finally delivered to various organelles for reception by copper-dependent proteins.[180] Copper transport proteins have been shown to be involved both in the cellular transport and in the resistance mechanisms of platinum-based chemotherapy.[181-183] Specifically, expression of human Ctr1 (hCtr1) is believed to cause an increased sensitivity to cisplatin, whereas expression of two Cu(I) proteins exporting ATPases (ATP7A and ATP7B) may be involved in the resistance mechanisms to platinum-based metallodrugs.[152, 180] Specifically, ATP7A can sequester the platinum containing species away from its targets, and ATP7B has been shown to excrete cisplatin from the cell.

hCtr1 is the main copper influx transporter in mammalian cells, and plays a significant role in the homeostatic regulation of the intracellular levels of copper to ensure the transport of copper to the demanding enzymes. hCtr1 is found in the plasma membrane and is constituted of three transmembrane helices, an extracellular N-terminal domain and a cytosolic C-terminal domain.[184] Two Met-rich and two His-rich motifs on the extracellular N-terminal domain have been demonstrated to be essential for the function of the transporter,[185] and the Met-rich regions (located in the N-terminal domain and in the inner side of the channel pore of the protein) were described as necessary for the binding and transport of

copper.[186] hCtr1 has also been characterised as playing a critical role in the cytotoxic activity of platinum drugs in cells.[185] Expression of hCtr1 in a cell line treated with cisplatin induced the formation of a stable homotrimer complex of hCtr1 (time and concentration-dependent) never observed following addition of copper to these same cells. Mutagenesis studies allowed the identification of two methionine-rich clusters in the N-terminal extracellular region of hCtr1 (^{7}Met-Gly-Met-Ser-Tyr-Met-Asp-Ser^{14} and ^{39}Met-Met-Met-Met-Pro-Met-Thr-Phe^{46}) necessary for the formation and stabilization of the multimer by cisplatin, thus suggesting their involvement in the binding to the metallodrug and potentially in the formation of crosslinks between hCtr1 peptide chains.[187]

In this context, ESI-MS experiments have been exploited by Natile *et al.,* in combination with NMR spectroscopy, to study the interactions of cisplatin and transplatin with a methionine-rich octapeptide used as a model for the regions involved in the multimer formation of Ctr1.[55] Cisplatin was shown to quickly form a mono-adduct of the type $[peptide+PtCl]^{+}$, and after 24 h incubation, no native apo-peptide was detected and the main specie was determined to be $[peptide+Pt(OH_2)]^{2+}$, eventually converting into $[peptide+Pt]^{2+}$. The loss of the ammine ligands was suggested to be indicative of the *trans* effect induced by binding to S-donor methionine residues, also responsible for the high reactivity of cisplatin towards this peptide. Conversely, when transplatin was incubated with the peptide in the same conditions, only an adduct of the type $[peptide+Pt(NH_3)_2]^{2+}$ was detected, increasing slowly overtime and remaining stable for days.[55]

Similarly, Franz *et al.* reported on the use of LC-MS to compare the reactivity of cisplatin, carboplatin and oxaliplatin towards two synthetic peptides representing the two methionine-rich sequences of N-terminal hCtr1 thought to be the potential binding sites of platinum.[188] Cisplatin was described as having the same reactivity pattern towards both peptides: after 4 h incubation, the molecular ion peaks of the apo-peptides were significantly reduced and the main adducts were found to be $[peptide+Pt]^{+}$, and, in the case of the ^{7}Met-Ser^{14} peptide, an additional adduct corresponding to $[peptide+PtCl]^{+}$. In each case, the binding sites were presumed to be three Met residues and a deprotonated amide nitrogen from the peptide backbone (only when the platinum centre lost its remaining chlorido ligand).[188] Similarly, oxaliplatin incubated for 4 h with the ^{39}Met-Phe^{46} peptide gave rise to $[peptide+(DACH)Pt]^{2+}$ and $[peptide+(DACH)PtCl]^{+}$ adducts. After 24 h, the main adduct was identified as $[peptide+2[(DACH)Pt]]^{4+}$.[188]

As expected, carboplatin was described as significantly less reactive towards the ^{39}Met-Phe^{46} peptide than cisplatin and oxaliplatin. After 24 h incubation, the only peak observed on the mass spectrum was the apo-peptide. However, after 48 h, adducts attributed to $[peptide-H+Pt]^{+}$ were also detected, demonstrating, as in

the case of cisplatin, complete loss of the original ligands.[188] Based on previous reports on the high reactivity of carboplatin with the model protein Cyt c, it may be possible that the affinity of the compound for the short hCtr1 model peptide is not representative of the "real" reactivity towards the intact protein.

Tandem MS techniques have been applied to identify the binding sites of different platinum-based compounds (cisplatin, transplatin, carboplatin and oxaliplatin) to a 20-mer peptide corresponding to the N-terminal domain of hCtr1.[189] Thus, ESI-MS revealed the ability of Pt(II) complexes to bind to the peptide in 1:1 and 2:1 ratio and identified methionine residues as the metal binding site(s). Furthermore, the study revealed that adducts' nature and stability were dramatically influenced by the nature of the original ligands in the metal complex, where the *cis*/*trans* geometry, type of leaving and non-leaving group(s) (*i.e. cis/trans* effect, labile or non-labile ligands) were playing a role. Indeed, while cisplatin and carboplatin were found to form only adducts where all the initial ligands were lost, transplatin and oxaliplatin formed adducts still containing the nitrogen donor ligands and reacted more rapidly with the peptide. More precisely, small amounts of mono-Pt adducts were detected after a few hours in the case of cisplatin and carboplatin, whereas mono- and bis-Pt adducts were observed after 5-30 min for both transplatin and oxaliplatin. In all cases, after 72 h incubation, the main adduct corresponded to a "naked" platinum ion bound to the peptide. A scheme of the overall reactivity is reported in Figure 8.

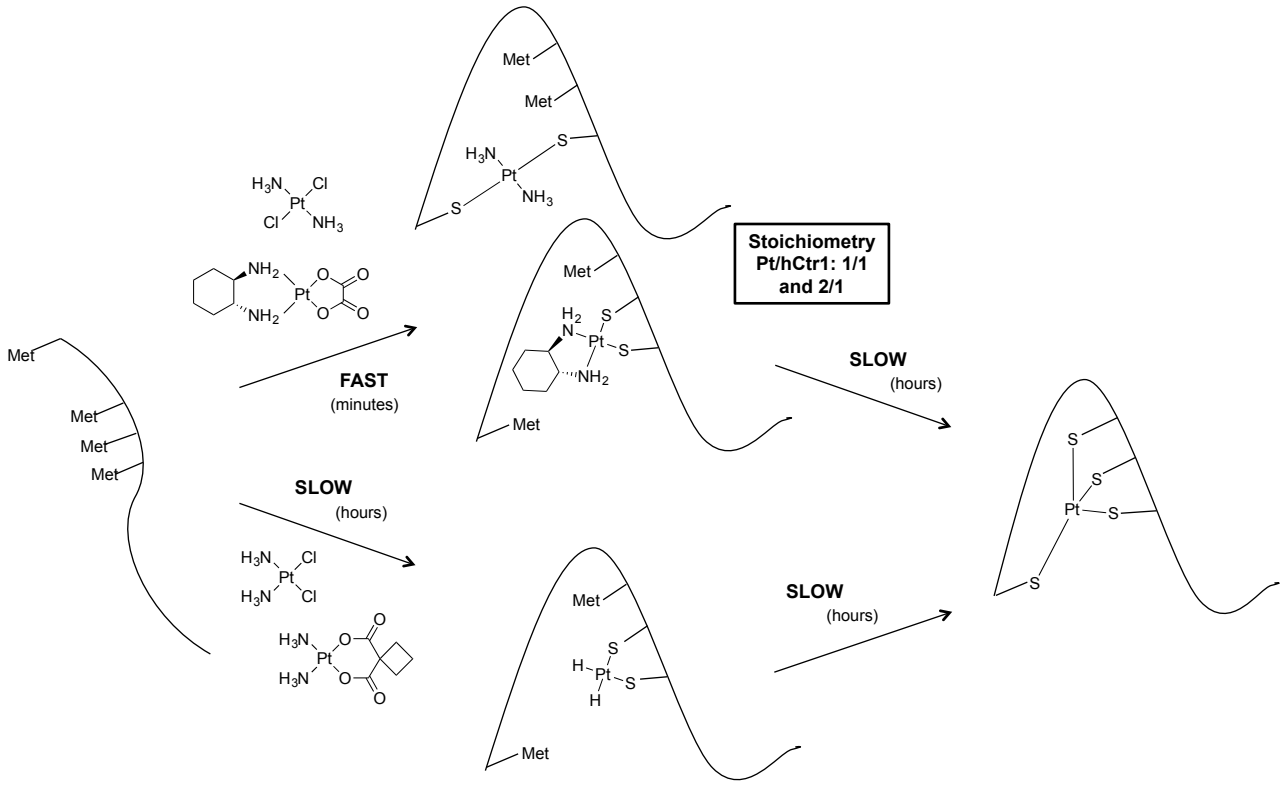


Figure 8. Schematic reaction pathway between the 20-mer peptide corresponding to the N-terminal domain of human copper transporter 1 (hCtr1-N20) and different platinum(II)-based drugs. Adapted from ref [189].

Interactions between platinum drugs and the chaperon protein Atox1 have also been studied by various techniques including mass spectrometry. Crystallised adducts of the protein incubated with cisplatin revealed the formation of a mono-adduct in which Cys12 and Cys15 coordinates the platinum centre with a *trans* geometry, the other ligands completing the square planar geometry being the amide nitrogen of Cys12 and an exogenous molecule of 2-carboxyethylphosphane (TCEP).[190] Natile *et al.* were able to describe in details the different steps for the coordination of cisplatin to Atox1 in physiological media using in-cell NMR spectroscopy,[191] and also combined NMR and ESI-MS to study cisplatin's interaction to the metal-binding domain of ATP7A (MNK1) and/or Atox1 in the absence or presence of GSH.[192] Cisplatin was found to form the expected first step chelate adducts with both Atox1 and MNK1 ($[protein+Pt(NH_3)_2]^{2+}$), with no apo-proteins detected after 24 h incubation. When the proteins were exposed to a 10-fold excess of glutathione prior incubation with the metallodrug, Atox1 was found unreactive towards cisplatin (with only cisplatin-GSH adducts detected in the mass spectrum), whereas MNK1 was able to compete with GSH for binding to cisplatin.[192] Interestingly, when Atox1 was used in its holo-form (pre-incubated with copper), despite the presence of GSH, Cu(I)-Atox1 was able to form adducts corresponding to $[Cu(I)\text{-}Atox1+Pt(NH_3)_2]^{2+}$ after 3 h.[192] When glutathione was incubated with the pre-formed platinum adducts, no release of platinum from the protein was observed. The presence of TCEP during incubation of Atox1 with cisplatin induced the formation of the adduct $[Atox1+Pt(TCEP)_2]^{2+}$, indicating the coordination of two phosphorus atoms from TCEP on platinum, in addition to two cysteine residues from the protein.[192]

The influence of copper binding on Atox1 with respect to the reactivity of cisplatin has been further investigated by Liu *et al.* using 2D NMR spectroscopy and ESI-MS.[193] Copper coordination to Atox1 promoted significantly the binding of platinum to the protein and prevented metal release upon incubation with reducing agents such as DTT.[193] The nature of the platination sites on both apo- and holo-Atox1 was investigated using a bottom-up approach. After tryptic digestion, the obtained fragments were then analysed using CID and the residues Cys12 and Cys15 were confirmed as the platination sites in both cases, as observed by X-ray crystallography.[190, 193]

Interestingly, to evaluate the reactivity of Pt(IV) prodrugs interactions towards copper transporters, two model peptides (the octapeptide Mets7 (Met-Thr-Gly-Met-Lys-Gly-Met-Ser), resembling to one of the Met-rich motifs present on the extracellular N-terminal domain of hCtr1, and MNK1, the first cytoplasmic domain of ATP7A) have been incubated and analysed by ESI-MS by Osella *et al.*[194] Both apo-peptides

were found to be unreactive towards the dicarboxylato Pt(IV) analogue of cisplatin even after 5 days of incubation, suggesting their inability to reduce Pt(IV) to Pt(II). Upon addition of an excess of reducing agents such as GSH and ascorbic acid, adducts corresponding initially to $[Mets7+PtCl]^+$ and then evolving into $[Mets7+Pt]^{2+}$ were observed, as in the case of cisplatin. It is worth mentioning that the reduced Pt(II) species underwent immediate loss of both ammine ligands, indicating most likely replacement by Met residues of the peptide.[194] In the case of MNK1, the addition of reducing agents led to the precipitation of the protein thus making the analysis by MS impossible. The main conclusion drawn for this study was that Pt(IV) prodrugs have a different reactivity than cisplatin towards copper transporters, suggesting a different transport mechanism, potentially rather *via* passive diffusion.[194]

4.2 Glutathione-S-transferase

Glutathione-S-transferases (GSTs) are a group of enzymes (divided in three major families: cytosolic, mitochondrial and microsomal) that has been identified by proteomics methods to be overexpressed in cisplatin-resistant human ovarian cancer cells compared to cisplatin-sensitive native cancer cells.[195-197] GSTs have been found to be involved in processes of cellular detoxification from toxic compounds of endogenous and xenobiotic sources.[198] For instance, the first step of detoxification pathway of mercapturic acid is assured by GSTs by conjugation of the reduced GSH to the electrophilic centre of the noxious compound.[199] Some cytosolic GST isoforms like P1-1 have gained attention in the recent years due to their emerging role in cancer development and drug resistance mechanisms.[200, 201] GSTs' active site is constituted by the G site (N-terminal domain of the protein and GSH binding site) and the H site (C-terminal domain and hydrophobic/electrophiles binding sites).[202-205] Two accessible cysteine residues - Cys47, near the G site and crucial for maintaining its stability and conformation, and Cys101, which is able to form a disulfide bridge with Cys47 - can affect the catalytic activity of the protein. In fact, the formation of the disulfide bridge leads to a conformational change of the protein and a deactivation.[206] Interestingly, Cys47 and Cys101 have been also found able to interact with metal ions such as iron.[207, 208]

Casini, Lo Bello *et al.* reported on the use of several techniques including MS to study the interaction between auranofin and GST P1-1.[209] Auranofin was found to inhibit the activity of the protein in the same IC_{50} values range than a known organic inhibitor (ethacrynic acid), and the molecular mechanism of inhibition was studied by ESI-FT-ICR-MS. After 30 min incubation with P1-1, adducts corresponding to [GST P1-1+$Au(PEt)_3$] and [GST P1-1+2($Au(PEt)_3$)] were detected and were found stable overtime, indicating that at

least two gold binding sites are accessible. The same experiments were repeated with mutants of the protein and revealed that an unidentified amino acid that is neither Cys47 nor Cys101 is the primary binding site of auranofin, and that Cys101 is most likely the second binding site of the metal complex.[209, 210]

Recently, Wang *et al.* reported on the study of the interactions between organometallic Ru(II) arene anticancer complexes and a specific GST isoforms (GSTπ) (whose activity is inhibited in presence of the ruthenium complexes) using MS techniques.[205, 211] GSTπ is widely distributed in the erythrocytes and tissues (e.g. lung, prostate), and overexpressed in multiple types of solid tumors.[210, 212-214] Furthermore, GSTπ possesses 4 cysteine and 3 methionine residues that can potentially be binding sites for the ruthenium complexes.[205] A bottom-up approach has been utilised to determine the binding sites of ruthenium on the protein using consecutive tryptic digestions and HPLC-ESI-MS.[205] The nature of the arene was shown to influence the nature and localization of the binding sites. When the selected arene was *p*-cymene, Cys15, Cys48 (from the G-site) and Cys102 were identified as the binding sites of [(η^6-p-cymene)Ru]$^{2+}$ and [(η^6-p-cymene)Ru(en)]$^{2+}$ fragments, and the examination of the crystal structure of the protein confirmed that those residues were available for binding.[205] If the arene was a biphenyl ligand, Cys48 and Met92 were identified as the binding sites, whereas Cys15, Cys48 and Met92 were preferred by the complex bearing a 9,10-dihydrophenanthrene.[205]

A complementary comprehensive study by HPLC-ESI-QToF-MS reported on the quantification of the binding stoichiometry of the same ruthenium complexes to GSTπ.[211] The results showed that pre-complexation of the protein with GSH significantly prevented the binding of the metal complexes towards Met92 and Cys102 residues but had little impact on the reactions with Cys15 and Cys48. As the inhibition of the protein by the ruthenium complexes was found similar independently of the pre-complexation or the absence of GSH on GSTπ, residues Cys15 and Cys48 were, therefore, considered as responsible for the enzyme inhibition properties of the ruthenium complexes.[211]

4.3 Zinc finger proteins

A zinc finger (ZF) is a small protein structural motif that is characterized by the coordination of one or more zinc ions in order to stabilize the fold. Proteins that contain zinc fingers (zinc finger proteins) are classified into several different structural families. They are widespread throughout the human genome and are part of the proteins requiring Zn^{2+} ions to maintain their appropriate secondary and tertiary structures and to perform their biological function.[215] Each class of ZF proteins is not only determined by its three-dimensional

structure, but also by the primary structure and the identity of the ligands coordinating the zinc ion. The majority of ZF proteins typically function as interaction modules that bind DNA, RNA, proteins, or other small molecules, and variations in structure serve primarily to alter the binding specificity of a particular ZF protein. In general, zinc fingers coordinate zinc ions with a combination of cysteine and histidine residues. Thus, Zn^{2+} binding is assured by four residues of the proteins (*i.e.* Cys_4, $Cys_2HisCys$ or Cys_2His_2 types of domains) generating a tetrahedral geometry around the metal centre, while the zinc finger domain is in general constituted by ca. 30 to 40 residues.[216] ZF proteins are the most common DNA binding motifs in human transcription factors, but are also present in DNA repair proteins, and in retroviral nucleocapsid proteins among others, and insure a wide range of various biological functions such as DNA recognition, protein folding, RNA conditioning, regulation of apoptosis and transcriptional activation.[152] [217, 218]

Release of Zn^{2+} or substitution by another metal ion, as well as mutation of the Zn binding sites, result in most cases in loss or malfunction of the zinc finger protein biological activity. Several metal-based compounds have been found able to displace Zn^{2+} from the ZF domains, therefore altering their activity.[216] For example, Casini *et al.* described the inhibition properties of various metal complexes towards the poly(adenosine diphosphate (ADP)-ribose) polymerase 1 (PARP-1), an essential ZF protein involved in DNA repair mechanisms and cancer resistance to chemotherapeutics.[106, 219, 220] PARP-1, possessing two ZFs at the N-terminal side, can recognize cisplatin-damaged DNA, having a stronger affinity for the cross-links induced by the metallodrug, and initiate the repair process.[221-225] Of note, PARP-1 inhibitors have been considered as a potential family of chemotherapeutic drugs in combination with DNA “alkylating” agents (e.g. cisplatin and carboplatin) to increase their efficacy in cancer cells, and they are currently in clinical trials.[226-230]

Within this framework, a number of cytotoxic gold-based complexes have been described as excellent inhibitors of PARP-1 (IC_{50} in the nM range) and MS techniques were used to identify the mechanism of inhibition at a molecular level.[219] Specifically, a first study reported on the interactions between the metal-based anticancer agents cisplatin, RAPTA-T, NAMI-A, auranofin and Auphen ($[Au(phen)Cl_2]Cl$ (phen = 1,10-phenanthroline)) with a 44 amino acids peptide representing the last N-terminal zinc binding domain of PARP-1 by high resolution FT-ICR mass spectrometry.[219] Interestingly, the extent of adducts formation was in good agreement with the protein inhibition values of the various complexes, with the gold complexes by far the most reactive. Thus, an excellent correlation between PARP-1 inhibition in cell extracts and the ability of the complexes to bind to the ZF motif (in competition with Zn^{2+})

was established by MS. The results supported a model whereby displacement of zinc from the PARP-1 ZF ($Cys_2HisCys$ type) by gold ions leads to decreased PARP-1 activity, and to the formation of the so-called ‘‘gold-finger’’ (GF). In this study, for the first time, it was possible to observe that Au(III) ions from the complexes maintain the oxidation state +III upon peptide binding, showing that if the metal centre can reconstitute its preferred coordination sphere (bound to 4 ligands) redox processes are not favoured. Notably, since the coordination geometry of Zn(II) ions in the ZF is tetrahedral, substitution with Au(III) ions should give rise to a square planar coordination which alters the overall folding and disrupts the hydrogen bond network essential for DNA recognition. This hypothesis has been supported by QM/MM calculations on GF model domains.[231] Farrell *et al.* also described a similar reactivity of Au(III) coordination complexes with ZF model peptides by MS,[232, 233] while Barrios and coworkers reported on GF formation by Au(I) studied by different spectroscopic methods.[234]

More recent studies, using ESI-Orbitrap FT-MS were performed to assess whether coordination of the gold(I) and gold(III) compounds to another zinc finger domain (Cys_2His_2), characteristic of several transcription factors, was also occurring *via* substitution of the Zn^{2+} ion, with subsequent formation of GF (Figure 9), and to determine the binding stoichiometry.[231] Overall, the results suggest the influence of different zinc coordination spheres (Cys_2His_2 vs. $Cys_2HisCys$) in the formation of gold fingers, with the domain of PARP-1 richer in Cys residues being the most reactive. The results are in line with previously reported ones pointing at the contribution of the nature of the ZF domain in the modulation of Au(I) compounds biological activity, although in the latter case, the investigated compounds belonged to the family of the Au(I) phosphine-*N*-heterocycles.[235] Interestingly, when ESI-MS was again applied, together with various other spectroscopic techniques, to assess binding of another Au(III) complex Auterpy ($[Au(terpy)Cl_2]Cl$ (terpy = 2,2’:6’,2”-terpyridine) with a Cys_4-type of ZF domain, the spectra showed reduction to Au(I) upon adduct formation, again demonstrating that the number of Cys residues influence the reactivity of the metal compound.

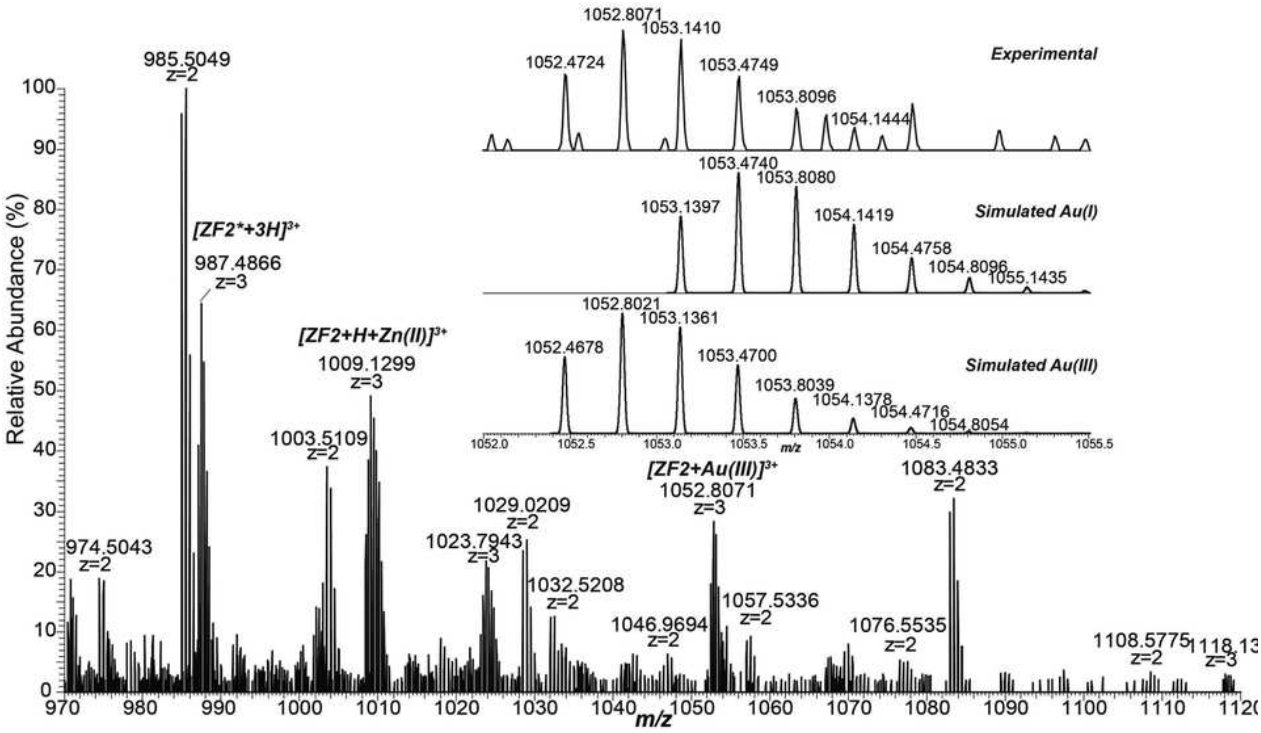


Figure 9. ESI-Orbitrap FT-MS spectrum of a reconstituted zinc finger model peptide (Cys_2His_2) incubated for 15 min with the cytotoxic gold(III) complex Auphen ([Au(phen)Cl_2]Cl (phen = 1,10-phenanthroline)). Gold finger formation is confirmed by the presence of the gold(III)-peptide adduct at *m/z* = 1052.8071. Reproduced from ref [231] with permission of the Royal Society of Chemistry.

Transcription factors, representing another important class of ZF proteins, have been studied using MS and other techniques for their reactivity towards metal-based compounds. Ralph *et al.* reported the ability of platinum and ruthenium-based complexes to modulate the activity of the transcription factor PU.1-DBD towards a double-stranded DNA molecule containing the sequence recognising the transcription factor by nESI-MS.[236] ESI-MS followed by tandem mass spectrometry (using a bottom-up approach) have been described to characterise the interactions between cisplatin and the apo- form of the breast cancer susceptibility protein 1 (BRCA1) RING finger domain.[237] Cisplatin was found able to affect the conformation of the protein, by forming inter- and intra-molecular Pt-BRCA1 adducts, and His117 was identified as the preferential binding. The same authors also studied the consequences of the platination of the BRCA1 RING domain by cisplatin and analogues *in vitro*, and observed the inhibition of the ubiquitin ligase activity of the protein.[238]

Farrell *et al.* used ESI-ToF-MS techniques to characterise the interactions between Au(III), Pt(II) and Pd(II) complexes bearing N^N^N tridentate ligands (diethylenetriamine or 2,2':6',2"-terpyridine) towards a model peptide of the C-finger of HIV nucloecapsid NCp7 (Cys_2HisCys type), which plays a critical role at different steps of the retrovirus life cycle.[232, 239] Both Pt and Pd complexes were found to have a similar

reactivity towards the peptide, with displacement of a chlorido ligand by a zinc-bound thiolate, followed by loss of the N^N^N ligand due to the strong *trans* influence of the thiolate, and finally ejection of zinc and generation of adducts with 'naked' Pd(II) or Pt(II). The Au(III) complexes were significantly more reactive, allowing direct detection of the GF domain, in which the gold ion had lost all of its initial ligands.[232] These results highlighted the potential of gold-based drugs as new class of anti-HIV agents based on the inhibition of the NCp7 function.

In conclusion, the overall of these results showed that zinc displacement from ZF proteins can be attained by coordination and organometallic compounds and modulated by the nature of the metal and the ligands to gain selectivity and to target specific ZF domains for different pharmacological applications.

4.4 Seleno-enzymes

Seleno-proteins, containing selenocysteine amino acid residue(s), have been identified throughout the human body, and have been shown to play critical roles in essential biological processes. Thus, selenium deficiency has been associated with a variety of human diseases including cancer. Two main seleno-proteins, thioredoxin reductase (TrxR) and glutathione peroxidase (Gpx), which contain a seleno-cysteine residue at their active site, have been characterized as involved in defence and repair mechanisms of oxidative damages.[240] TrxR has been shown to catalyse, in a NADPH dependent manner, the reduction of thioredoxin to counter-balance the oxidative processes, whereas Gpx was identified as reducing peroxides to their corresponding alcohols using glutathione as the electron donor.[241-243] Taking in consideration the role of oxidative stress in carcinogenesis, it is most likely that adapted expression and activity of these proteins appear as essential in cancer prevention.[244] Furthermore, a few examples of selenium-based compounds exerting anticancer activity have been reported.[245]

Interestingly, recent research focused on targeting TrxR for cancer treatment.[240, 246, 247] Both cytosolic (TrxR1) and mitochondrial (TrxR2) isoforms are essential regulators of the redox balance and participate in a variety of functions (e.g. DNA repair, cell proliferation, angiogenesis, transcription).[240] It is also well established that TrxR is involved in cancer cell proliferation, as several cancer cell lines exhibit overexpression of the enzyme, which can also be associated with resistance to chemotherapies.[240] The C-terminal sequence containing the seleno-cysteine (Sec) residue has been identified as reactive towards several compounds, and such interaction was shown to inhibit the activity of the protein. Although cisplatin and gold-based (Au(I) and Au(III)) complexes have been described to inhibit TrxR using *in vitro* assays,[248-

258] so far only a few studies focused on determining the stoichiometry of the binding or are aimed at identifying the binding sites of metal-based inhibitors. As an example, MALDI and ToF-MS experiments were reported as efficient methods to quantify the stoichiometry of the binding in spite of the relatively large size of the protein (≈110 kDa for the homodimeric protein).[248] Upon incubation of the protein with an excess of auranofin, extensive metallation was noticed, with up to 4 $[AuPEt_3]^+$ bound fragment, indicating that a large number of binding sites are available for gold atoms, not only the Sec residue. A complementary study by Casini *et al.* showed a similar significant reactivity of other gold-based compounds towards TrxR1, both for Au(I) (auranofin, aurothiomalate) and Au(III) ($[Au(dien)Cl]Cl_2$ (dien: diethylenediammine, $[Au(bipy)(OH)_2][PF_6]$ (bipy: bipyridine), $[(Au(py^{dmb}\text{-}H)(AcO)_2]$ (py^{dmb}:2-(1,1-dimethylbenzyl)-pyridine, AcO: acetate), $[Au(bipy^{dmb}\text{-}H)(2,6\text{-xylidine-H})][PF_6]$ ($bipy^{dmb}$: 6-(1,1-dimethylbenzyl)-2,20-bipyridine)) complexes, with almost a quantitative binding (e.g. up to 10 equivalents of gold adducts bound upon incubation with an excess of gold complex), confirming the presence of a multitude of potential binding sites other than Sec accessible on the protein.[258] A bottom-up approach using tryptic digestion followed by MS/MS experiments was then used to determine the residues involved in gold binding. The peptide fragment $^{236}I\text{-}K^{246}$ was found to bind one Au(I) ion most likely coordinated to one of the histidine residues of the peptide, although identification of the exact binding sites was not possible by MS.[258]

The compound $[(PPh_3)AuCl]$ was also tested towards a cysteine-containing model peptide of TrxR1 and the nature of the adducts determined by hyphenated LC-ESI tandem mass spectrometry experiments.[259] In this case, covalent binding was detected and Cys identified as the anchoring site of the 'naked' gold ion, having lost the chlorido ligand followed by loss of the phosphine.[259] Messori *et al.* also reported on the use of a model peptides representing the C-terminal motif of human TrxR to study the interactions with auranofin and another cytotoxic dinuclear *μ*-oxo Au(III) complex by ESI-MS.[260, 261] The tetrapeptide Gly-[Cys-Sec]-Gly (S-Se bound), matching exactly the C-terminal sequence of the protein, was shown to be unreactive towards all the Au complexes in the absence of reducing agents. Upon pre-reduction of the peptide with DTT, auranofin was found to bind tightly with loss of its tetraacetate thioglucose ligand, and retention of the PEt_3 moiety, and MS/MS experiments revealed that the Sec residue is the preferential binding site.[260] Interestingly, incubation of the peptide with $NaAuCl_4$ and the dinuclear *μ*-oxo Au(III) complex gave rise to the same type of adducts, *i.e.* DTT + 2 Au(I) + peptide, with reduction of the initial Au(III) in both cases, and preferential binding at the Sec residue.[260] It should be noted that, in this case, gold reduction may be induced by the presence of DTT and may not occur in physiological conditions.

Another model peptide of 11 amino acids containing the C-terminal fragment (and the Se-Cys residue) of hTrxR was also described by the same authors for its reactivity towards a series of Au(I) *N*-heterocyclic carbene (NHC) compounds using ESI-MS.[261] Specifically, the compounds featured, on one side, the same carbene ligand, *i.e.* 1,3-diethylbenzimidazol-2-ylidene, while as ancillary ligand either a chloride, or a second NHC ligand (identical to the first one), or triphenylphosphine. In all cases, adducts formation involved the presence of Au(NHC)+peptide species, where retention of the NHC ligand by the Au(I) ions was observed. The study also revealed that stable adducts were formed between the peptide and the gold carbene complexes, although the degree and rate of adducts formation was influenced by the second ancillary ligand of the gold complex. A top-down MS^2 experiment confirmed the preferential binding site to be the Se-Cys residue, with the NHC ligand still bound to Au(I), while cysteine could act as a secondary binding site.[261] A different reactivity was evidenced for a terpyridine-platinum(II) complex, as reported by Wang *et al.*, whose binding site on TrxR1 were sulfur donors in the GlyCysCysGly motif as revealed by different analytical methods including tandem mass spectrometry.[262]

Arsenic trioxide (As_2O_3), a compound used for centuries in traditional medicines and which has recently been considered for treatments of leukaemia and solid tumors, has been identified as a potent inhibitor of TrxR.[256, 263] Holmgren *et al.* reported on the use of a bottom-up approach and MALDI to identify the binding sites of As on the protein.[263] The results suggested that arsenic trioxide binds to both sulfur and selenium in the C-terminal motif GlyCysSecGly of the protein, and although precise identification of the coordination sites was not possible, the N-terminal sequence was also found to participate in the reaction with As.[263] However, it is worth mentioning that in this report, no evidence for the selenium isotopic pattern on the peptide fragment bound to arsenic has been provided.

4.5 Metallothioneins

The major intracellular thiols involved in metallodrug resistance are glutathione (GSH) and metallothionein (MT). In this review, we have included the metallothioneins in the section dedicated to protein targets, since these proteins can be considered as “negative” but still likely targets for metallodrugs, affecting their pharmacological activity. In details, MTs are small cysteine- and metal-rich proteins that have been identified as playing a critical role in the acquired resistance mechanism of platinum-based chemotherapy.[264] Thanks to their high number of cysteine residues, they are able to bind up to 7 metal ions at a time, including zinc, copper and cadmium, thus making them an important storage site for some essential elements and a

powerful detoxification system.[265] Human MTs are composed of a single polypeptide chain of 61–68 amino acids including 20 cysteines. The cysteine thiolates are involved in the binding of 7 divalent metal ions forming 2 independent metal–thiolate clusters in which each metal is tetrahedrally coordinated by both terminal and bridging thiolate ligands. Naturally occurring MTs usually contain 7 Zn(II) ions. From the four MTs expressed in humans (designated MT-1 through MT-4), MT-1 and MT-2 occur ubiquitously in high amounts in mammalian cells. In contrast to MT-3/-4, the biosynthesis of MT-1/-2 is inducible by a variety of compounds including hormones, cytokines, and metal ions. Both inducible MTs (MT-1/-2) play a role in resistance to antitumor platinum drugs.

A variety of analytical methods have been described to characterise the interactions between cisplatin and MT using *in vitro* and *in vivo* models, suggesting that cisplatin binding occurs by ejection of both cadmium and zinc from the native protein.[265] However, nanospray tandem QToF-MS and SEC-HPLC-ICP-MS experiments were described by Li *et al.* on a native rabbit MT containing 1.4% zinc and 7.9% cadmium, showing that only zinc was ejected during reaction with cisplatin, while cadmium remained bound to the protein.[265] Tandem MS analysis of the adducts revealed for both cadmium and platinum preferential bindings on cysteine residues, but other residues could not be excluded for platinum.[265]

Vasak *et al.* reported on comparative studies of *cis*- and *trans*-based platinum complexes towards both Zn_7MT-2 and Zn_7-MT-3 using mass spectrometry amongst other techniques.[120, 264] The difference between those two MTs is that the biosynthesis and expression of MT-2 are triggered by metal ions (amongst other compounds), whereas MT3 is not inducible.[120] In the case of MT-2, results showed that *trans*-platinum complexes react faster than the *cis*-ones. Characterization of the adducts revealed that in the case of the *cis*-Pt(II) compounds, all initial ligands were replaced by cysteine residues, whereas *trans*-Pt(II) complexes were found to retain their N-donor ligands, thus remaining in a potentially biologically active form.[120] The steric hindrance of the ligands as well as the electrophilicity of the platinum centre were also pointed out as influencing the kinetics of the reactivity of replacement of the zinc ions by platinum.[120] Using MT-3, a similar trend was reported, transplatin reacting faster than cisplatin, and confirmation of the release of a stoichiometric amount of Zn from the peptide upon Pt binding was shown.[264] As in the case of MT-2, transplatin was shown to retain its ammonia ligands, while cisplatin lost all of its initial ligands, replaced by cysteine thiolates. However, since MT-3 is, in some cases, overexpressed in cancer tissues and not regulated by the presence of heavy metals, the conclusion drawn from a mechanistic point of view of this

study was that platinum-based metallodrugs administered would be kinetically favoured to bind to MT-3, and the induced release of zinc in this process would upregulate the expression of MT-2.[264]

Similar reactivity studies of ruthenium-based metallodrugs towards MTs using MS techniques have been reported. Casini *et al.* studied the interactions of the organometallic RAPTA-C complex towards rabbit MT-2 (very similar to human MT-2) using ESI-MS and ICP-AES.[266] ESI-MS experiments determined that only ruthenium mono-adducts were formed, with the ruthenium centre still bearing the η^6-p-cymene ligand and in some case the pta. Release of zinc was noticed, as in the case of cisplatin, and quantified using ICP-AES. Overall, the results shown that RAPTA-C had a lower affinity for the protein than cisplatin, potentially explained by the fact that the *p*-cymene ligand is still bound thus creating steric hindrance, but that both complexes required ejection of zinc to react, suggesting that the cysteine binding sites were the same for both compounds.[266] Wang *et al.* also reported on the use of MALDI-ToF-MS experiments to study the interactions between organometallic $[(\eta^6\text{-arene})Ru(en)Cl]^+$ complexes and MT-1 and MT-2.[267] At neutral pH, the complexes were found significantly less reactive than cisplatin towards both proteins, and the interactions were even completely absent at lower pH, which could explain partly the lack of toxicity of this class of compounds, and a lack of cross-resistance to cisplatin. A tryptic digestion was attempted on the proteins incubated with the metallodrugs, however, no binding sites were identified.[267]

To the best of our knowledge, no studies report on the interactions of gold-based anticancer agents towards MTs. However, Wang *et al.* compared the interactions of several noble metals ions towards MT-2 using SEC-ICP-MS, RP-HPLC-ESI-MS and MALDI-ToF-MS techniques and prove that Au^{3+} ions were able to react quickly with the protein, with stoichiometric ejection of zinc.[268] Nevertheless, it is worth reminding that the reactivity of the metal complexes could differ from those of free metal ions, so no conclusion can be drawn concerning their reactivity in a biological context.

4.6 DNA G-quadruplexes

DNA can adopt different structures besides the canonical right-handed double helix (B-DNA). Crystallographic and NMR studies have revealed that guanine-rich DNA sequences can form quadruply-stranded structures, named G-quadruplexes, some of which are biologically relevant.[269] G-quadruplexes (G4s) are specific architectures of nucleic acids adopted by guanine-rich DNA and RNA sequences, whose stability comes from the stacking of contiguous G-quartets (planar and cyclic association of four guanine residues mediated by a central potassium or sodium ion in a Hoogsteen H-bonding arrangement).[270] G-

quadruplexes are currently widely studied as they are suspected to play a crucial role in key cellular mechanisms. Indeed, G4 structures have been identified in eukaryotic telomeres and in promoter regions of some oncogenes.[271, 272] Interestingly, the formation of quadruplexes causes a net decrease in the activity of the enzyme telomerase, responsible for maintaining the length of telomeres. The formation and/or stabilization of G-quadruplexes by selective small molecules, so-called G-quadruplexes ligands (stabilizers) appear as a novel therapeutic strategy to control those key cellular events.[269, 272, 273]

Most of the molecules reported to date as quadruplex DNA stabilisers/binders are based on large organic heteroaromatic systems. However, a number of metal complexes have also been designed to bind G4s.[274] Thus, anticancer platinum-based complexes were among the first to be investigated for their selectivity and reactivity towards G-quadruplexes structures, as DNA has already been identified as their main pharmacological target. Chottard *et al.* reported in 2003 the use of a bottom-up MALDI-ToF approach combined with gel electrophoresis migration experiments to quantify and assign the platinum binding sites on quadruplex structures of two human telomere sequences in presence of Na^+ and K^+ upon incubation with cisplatin and transplatin.[275] Mono- and bis- (cross-linking) platinum adducts were identified on both quadruplexes structures and in presence of both sodium and potassium, each platinum atom being most likely anchored *via* two remote purine of the sequences. Cross-linking of the purines from the quadruplex structures was hypothesised as preventing structured single-stranded telomerase sequences from unfolding and as potentially inhibiting telomerase activity.[275]

Berezovski *et al.* reported on the use of kinetic capillary electrophoresis (KCE) combined to IM-MS to study the conformational dynamics of DNA G4s in solution upon incubation with cisplatin.[276] In this case, cisplatin was shown to form strong intra-strand cross-links and to strongly influence the G-quadruplexes activity, however no selectivity for the G4s over other sequences was defined.[276] Tandem mass spectrometry using CID fragmentation experiments were reported by Schürch *et al.* to explore structural modification of mono-, bi- and tetra-molecular quadruplexes exposed to cisplatin.[277] By comparison with CD experiments, it was concluded that peaks of un-annealed single strands could not be attributed to decomposition of the structures upon ionization but rather to incomplete quadruplex formation prior to the measurements. For each of the three types of quadruplexes, exhibiting different structural features, the platination stoichiometry and rate of binding were found to be very different. The tetramolecular and bimolecular structures were found to bind a single intact cisplatin molecule, whereas multiple platination was observed for the monomolecular structure. Upon fragmentation, the platinated adduct of the

tetramolecular structure was found to release an unplatinated single strand. For the bimolecular structures, strand separation appeared as the main dissociation pathway. Identification of the platination sites on the monomolecular structure was possible, and the terminal G-repeats sequences were determined to be the favourable metallation sites.[277]

Interestingly, Vilar, Gabelica *et al.* reported on two novel [2+2] metallo-assemblies (-rectangles) based on a guanosine-substituted terpyridine ligand coordinated to either palladium(II) or platinum(II). The ability of these dinuclear complexes to interact with quadruplex and duplex DNA was investigated by a number of methods, including fluorescent intercalator displacement (FID) assays, fluorescence resonance energy transfer (FRET) melting studies, and high-resolution ESI-MS.[278] These studies have shown that both these assemblies interact selectively with quadruplex DNA (human telomeric DNA and the G-rich promoter region of c-myc oncogene) over duplex DNA. Specifically, the MS studies confirm that the compounds interact with quadruplex DNA and, particularly in the case of the platinum(II) rectangle, the dimetallic structure is retained upon binding. Moreover, based on MS data, Pt(II) favours dimerization of G-quadruplex structures.

Although most of the anticancer gold-based compounds have low affinity for binding to DNA, recently Casini *et al.* have identified organometallic gold(I) bis-NHC (*N*-heterocyclic carbenes) complexes (Figure 10) as potent and selective G4s stabilizers.[279] Notably, the chemical features of the mentioned compounds fully fulfil the basic requirements for an ideal G4 ligand: i) they are planar; ii) positively charged and thus prone to electrostatic interactions with negatively charged DNA; iii) have two aromatic caffeine ligands (*i.e.* a guanine analogue) which can associate to guanines of G4s through π-stacking interactions. Of note, a joint ESI-MS and X-ray diffraction (XRD) study unravelled the gold complex-G4 adducts at a molecular level.[280] Specifically, complex $[Au(9\text{-methylcaffein-8-ylidene})_2]^+$ (Figure 10) was reacted with a model G4 structure (arising from Tel23 sequence) and adducts formation was analysed by ESI-MS, revealing the presence of mono-, bis- and tris-adducts of the intact Au(I) complex with one equivalent of the DNA sequence accompanied by ammonium ions, guaranteeing the maintenance of the quadruplex structure.[280] XRD data confirmed the ability for the quadruplex to bind three Au(I) complexes in two different sites, where stacking interactions drive non-covalent binding of the metal complex.[280]

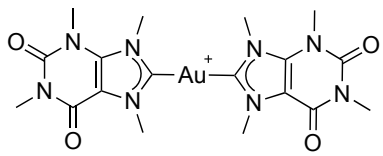


Figure 10. [[Au(9-methylcaffein-8-ylidene)$_2$]$^+$ cation as G4 stabilizer.

5. MS analyses of metallodrugs in complex biological systems

Metallomics and proteomics techniques have been extensively developed in the last decades to study the behaviour of metallodrugs in complex biological systems. In the proteomics approach, hyphenated chromatography and multidimensional separation techniques have allowed rapid and powerful identification of proteins compositions of organisms with known genome sequences.[281] The development of parallel metallomics techniques (to study of the interaction of metal-containing species with biological systems) contributed extensively to a better understanding of the behaviour of metal-based compounds administered in cells and in animal models.[282, 283]

Inductively coupled plasma mass spectrometry (ICP-MS), which can be coupled online to separation techniques such as capillary electrophoresis (CE) or liquid chromatography (LC), has played a crucial role in the investigation of metal-based drugs biodistribution, reaching high selectivity and sensitivity, especially for metal elements. Thus, ICP-MS is commonly used to determine the pharmacokinetics of metallodrugs, *i.e.* localization, distribution, metabolization and excretion of the complex, often in cells, blood or organs.[284] For instance, the total platinum content in rat organs and tissues was determined by ICP-MS after treatment of the animal with cisplatin, oxaliplatin and carboplatin for up to 90 days.[285]

Hyphenation of ICP-MS with laser ablation (LA) has been developed extensively in the past decade, in order to map the spatial distribution of the metallodrug inside tissues[286] or spheroids (3D models of tumors).[287] Such approach allowed for instance the visualization of the accumulation of cytotoxic metals in healthy tissues and organs and follow-up of the nephrotoxicity upon administration of cisplatin.[288, 289] Unfortunately, ICP-MS, although enabling accurate and absolute quantification of metals independently of their speciation in complex biological systems, presents a main drawback, as molecular information is lost due to the atomization of the sample.[283] Therefore, in this review we have chosen to discuss only those studies on complex samples that take advantage of molecular mass spectrometry techniques, although combined to ICP-MS.

In pioneering studies, a combined LA-ICP- and ESI-MS approach was reported by Allardyce *et al.* in 2001 to analyse cisplatin-treated *E. coli* cell extracts.[290] After extraction and separation of the proteins by 1D polyacrylamide gel electrophoresis gel electrophoresis (PAGE), the gel lanes were analysed by LA-ICP-MS for their metal content. The protein OmpA (Outer Membrane Protein A), involved in transmembrane

penetration of small solutes (and potentially cisplatin), was unambiguously identified by ESI-MS and MS/MS (acquisition of the peptide fingerprint followed by protein sequencing) as a main target for the metallodrug.[290]

Later on, LC-ESI-MS^2 has been used mainly to described the nature of the adducts and the binding sites of platinum-based drugs on DNA, as mentioned earlier in sections 2.2 and 4.6.[291] However, the technique was proven unable to detect interstrand DNA cross-links, which are highly cytotoxic and more difficult to remove by classical DNA repair mechanisms.[283, 292] SEC-ICP-MS techniques were also described to analyse the intracellular fate of the platinum and ruthenium-based metallodrugs, as well as the detection of metal binding partners, in HT-29 and A2780 cancer cells.[293, 294] For instance, Jarocz *et al.* reported on the analysis of KP1019-treated cytosol of cancer cells using SEC-ICP-MS.[294] The results suggested than 85% of the drug was converted into a high molecular mass fraction. A shotgun approach, with the entire proteome being digested and analysed by HPLC-MS^n was then used to further determine the ruthenium binding pattern, with both Ru(III) and Ru(II) protein adducts detected.[294] Overall, 15 proteins were detected in the cytosol of cancer cells and assigned as potential binding partners for the ruthenium species released from the adduct. Of note, certain proteins are bound to the Ru(II) species, while others prefer the Ru(III) species as binding partners. Proteins responsible for the reparation of DNA (BRCA1) and inhibition of apoptosis (APC) are found to form adducts with Ru(III) species, while Ru(II) species interact with pro-apoptotic proteins which cause apoptosis by the mitochondria pathway, namely cytochrome c and Apaf1. Such a type of coordination confirms the opinion that the drug “kills” tumors by a mechanism other than via direct interaction with DNA. Furthermore, it appears that only hydrolyzed forms of the drug can interact with cytosolic proteins, what is in accordance with literature findings. Next, the majority of peptides are coordinated to the Ru species containing no or a single indazole ligand.

Another example, described by Groessl *et al.*, demonstrated the value of SEC-ICP-MS to identify the binding partners of three metal-based drugs (cisplatin, NAMI-A and KP1019). Binding of KP1019 to high molecular weight proteins in the cytosol was first noticed by SEC-ICP-MS, followed by redistribution to lower molecular weight proteins after 24 h. Notably, the results showed that the targets of KP1019, even though not clearly identified, are located in the cytosol and not in the nuclei, confirming a distinct mode of action with respect to Pt(II)-based drugs.[293]

Interestingly, MALDI-ToF imaging was applied to study the distribution of Pt and Au in tissue slices.[295, 296] For instance, Larroque *et al.* described this technique on slices of rat kidney treated with

oxaliplatin, and concluded that the compound, unlike cisplatin, was mainly accumulating in the cortex and was thus not able to reach deeper penetration into the organ, explaining its reduced nephrotoxicity compared with cisplatin.[296] The same authors also reported on the use of a combined MALDI and LA-ICP-MS approach for Pt imaging in human tumor samples (colorectal and ovarian peritoneal carcinomatosis) of patients treated with cisplatin and oxaliplatin.[297] LA-ICP-MS was found to offer sensitive detection of Pt independently of the initial drug used or the sample matrix, whereas MALDI imaging was found to suffer in some cases from false negatives due to signal suppression by the matrix. More precisely, the results obtained with both techniques were coherent in the case of oxaliplatin, overall showing scarce penetration of the drug in the tissue, whereas in the case of cisplatin, metallation sites detected by LA-ICP-MS could not be observed by MALDI experiments.[297] The species monitored by MALDI MS was exclusively the monomethionine conjugates of oxaliplatin.

A bottom-up approach, allowing a comprehensive analysis (both metallomic and proteomic) of biological complex systems in a single experiment has been recently developed, based on the Multidimensional Protein Identification Technology (MudPIT). As previously mentioned, this technique is based on hyphenation of 2D liquid chromatography (reversed phase and strong cation-exchange (SCX)) and ESI-MS^n and allows up to 1500 proteins to be analysed in 24 h.[283, 298] This approach was first used to identify the cisplatin's binding sites in human serum proteins after tryptic digestion, revealing the coordination of the platinum atoms to sulfur- (cysteine and methionine) and carboxyl/hydroxyl-containing amino acids (aspartic acid, glutamic acid, tyrosine, serine, threonine).[155] MudPIT was also described for the first comprehensive proteomic analysis of proteins platination sites in the model organism *E. coli* treated with cisplatin.[39, 299] Thus, 31 proteins targets of cisplatin were identified thanks to this technique, such as MazG (nucleoside triphosphate pyrophosphohydrolase), mutS (DNA mismatch repair protein), DNA helicase II, topoisomerase I, efflux proteins and redox regulators, and possible platination sites were hypothetised. Nucleophilic O-donors such as aspartic acid, glutamic acid, serine, threonine or tyrosine were identified as metallation sites in 18 proteins, and methionyl S-atoms targets were found to bind to cisplatin in 9 proteins.[39] The same approach was described to analyse a Ru(II) complex which is not a drug candidate itself but represents a model for pharmacologically relevant Ru(II) arene class of compounds.[300] The results showed that the complex binds to cold-shock proteins that regulate stress response proteins as well as a DNA damage-inducible helicase. The main binding sites for the ruthenium fragments were identified as aspartic acid, lysine and threonine.[300]

Finally, a combination of MudPIT and metallomic studies was reported by Wolters *et al.* to characterize the effects of the ruthenium-based RAPTA-T drug towards human cancer cells.[301] First, a subcellular fragmentation was realised and the obtained fragments analysed by ICP-MS. Ru(II) was found to accumulate in the particulate containing the organelles rather than in the cytosolic, nucleic or cytoskeleton fractions. The fraction containing the most of the drug was then analysed by SEC-ICP-MS, and the results showed that ruthenium atoms were mainly bound to high molecular weight proteins in the cancer cells line sensitive to cisplatin, whereas in the cancer cells resistant to cisplatin, RAPTA-T was predominantly found in lower molecular weight species.[301] These results highlighted a different metabolism of the ruthenium-based drug depending on the sensitivity of the cancer cells to cisplatin. Complementary experiments with label-free protein quantification study revealed that 74 proteins were deregulated upon incubation with RAPTA-T (up- or down-regulation), shading new light on the cellular response mechanisms to metallodrug treatment.[301]

Recently, the use of nanoscale secondary ion mass spectrometry (nanoSIMS), which consists in a nanoscopic scale resolution imaging technique, was reported to localise Au- and Pt-based metallodrugs in tumors.[302, 303] Keppler *et al.* first reported the use of a combined LA-ICP-MS and nanoSIMS approach in complex biological systems (kidney and tumour samples) after treatment with Pt(IV) pro-drugs.[303] The results revealed that uneven platinum distribution was occurring in both the organ and subcellular fractions. LA-ICP-MS experiments allowed to localize the drug in the kidney cortex rather than in the medulla, and this section was submitted to nanoSIMS experiments. Sulfur-rich organelles in the cytoplasm were identified as the targets of the platinum drug, both in kidney and tumour cells. The malignant organelles taking up the drug were found of lysosomal origin, demonstrating the potential of this combinatorial approach in complex biological systems.[303]

Finally, nanoSIMS was also described in combination with EFTEM (Energy Filtered Transmission Electron Microscopy) to visualise a gold(I) phosphine complex in tumour cells.[302] This approach allowed the identification of sulfur-rich regions in the nucleus and in the cytoplasm to be the preferential binding sites of gold, supporting the hypothesis that the mechanism of action of such Au(I) anticancer agents is based in the inhibition of thiol-containing protein families such as TrxR.[302]

6. Conclusions and Perspectives

Understanding the mechanism of action of metal-based drugs to design optimised derivatives is challenging and requires the identification of the biological targets as well as the characterization of the compounds' reactivity at a molecular level towards different cellular components, including nucleic acids, protein constituents and other biological nucleophiles. At variance with organic drugs, research in this field has to focus on characterizing the various possible metal-containing species that are critical to activity - the "active species" of each metal compound - and the details associated with their specific nature and geometry. In terms of metabolism, metal-based therapeutics will generate multiple and variable chemical entities resulting from hydrolysis and ligand exchange reactions, as well as from redox processes, induced by the presence of a plethora of possible target biomolecules. Thus, the problem of metallodrugs' biological *speciation* needs be carefully addressed in order to fully benefit of the *prodrug* properties of metal complexes to achieve targeted pharmacological effects, while reducing toxicity due to uncontrolled reactivity. Noteworthy, in recent years, in order to control metallodrug's speciation, in addition to the development of various families of organometallic compounds featuring intrinsic enhanced stability due to the presence of metal-carbon bonds, some examples were reported of nano-formulation of anticancer metal-based complexes, including cisplatin (e.g. lipoplatin),[304] iron [305] and copper complexes.[306] Such type of drug delivery systems are ideal to reduce metal speciation and should find more applications in the future at the earliest stage of the metallodrug development process.

Among the possible investigational methods, mass spectrometry occupies a fundamental role to study metallodrugs speciation, as demonstrated by the various example discussed in this review, due to its versatility, possibility to be coupled to separation methods, relatively fast analysis of the samples, and presence of the analyte in physiologically relevant conditions. Certainly, mass spectrometry, since its invention a century ago, has evolved and adapted itself to various application domains at the interface between analytical, synthetic, medicinal and environmental chemistry to biology and biochemistry.[307] In this review we have provided an overview of the most widely studied therapeutic metal compounds *via* this powerful technique and tried to summarize the main outcomes for each selected case. Remarkable is the case of the organometallic Ru(II) complexes termed RAPTAs, which have been studied applying different mass spectrometry approaches to relatively simple samples (e.g. model nucleobases, amino acids, oligonucleotides and peptides) as well as to complex cellular systems (cell extracts). Overall, based on the obtained results on the speciation of RAPTA complexes, the current understanding of the role of each part of

the compounds' structure - *i.e.* the metal itself, labile ligands included, the pta ligand and the η^6-arene - modification of the aromatic ligand appears to be most promising for the development of targeted RAPTA-type drugs. The main limitation of this approach is that the arene ligand in RAPTA compounds is most readily derived from cyclic dienes, which necessitates somewhat elaborate synthetic procedures. Preliminary mechanistic studies using combinations of LA-ICP MS and ESI-MS, as well as exploiting the MudPIT technology, have started highlighting different proteins and low molecular weight fraction components, as possible targets for the ruthenium(II) complexes. In spite of these promising results, further MS studies, combined to *metallomics* approaches, are certainly necessary to progress in the investigation of the mechanisms of action in cancer cells.

Concerning future possible developments, if we consider that the tumor-inhibiting potential of new generation platinum metallodrugs is often estimated based on their (time-dependent) DNA reactivity, fast and reproducible analysis of samples *via* MS methods would be ideal to screen a wide number of compounds and select the best drug candidates. Indeed, as shown in this review, mass spectrometry was successfully applied to characterize the molecular reactivity of different experimental metallodrugs and to relate it to their biological effects, particularly where forming a coordination bond is part of the compound's mechanism of action. Nevertheless, in order to avoid biased conclusions, each experimental setup should be carefully selected and fine-tuned to exclude gas-phase interferences, and various types of samples, with increased degree of resemblance to physiological conditions in terms of components and their relative concentrations, should be analysed. In general, the utility of ESI-MS for structural characterization can be significantly enhanced when used in combination with two or more stages of mass analysis, *i.e.* tandem mass spectrometry.

In perspective, we are convinced that mass spectrometry should/could be applied and developed also to the investigation of metal compounds whose reactivity with biomolecules is based on "labile" coordination bonds (e.g. vanadium, copper complexes and other metal ions of the first row transition series) or on non-covalent interactions, such as those established by substitution-inert complexes including Pt(IV) and multinuclear Pt(II) compounds.[12, 14] Moreover, if adequately optimized, MS methods could be exploited to screen the binding of structural metal complexes to DNA G-quadruplexes and possibly identify selectivity profiles with respect to duplex DNA. Finally, mass spectrometry imaging methods should be further developed to study the reactivity and distribution of metallodrugs in complex biological samples, such as tissue specimens.

It is worth mentioning that in general, when exploring the metallodrug-biomolecule interactions, the fundamentals of organometallic and coordination chemistry must be applied as well as adapted. Indeed, most of the metal-based agents exhibit their anticancer properties after coordinative or covalent bonding with their biological target(s), based on the principle of hard and soft acids and bases (HSAB theory), even though the binding can also be highly affected by the surrounding microenvironment, also determined by the overall structure of the biological target (e.g. the protein isoelectric point, pH, hydrogen bond and electrostatic interactions, dielectric constant of the binding pocket etc.). Therefore, new chemical rules have to be defined to achieve the complete understanding of metallodrugs' (and more in general metal complexes') interactions in biological systems. In our opinion, this is one of the greatest challenges of modern inorganic chemistry (coordination and organometallic), which may add new trends to the periodic table aimed at predicting the reactivity of metal compounds in physiological environment. We are convinced that mass spectrometry, coupled to other spectroscopic and biophysical techniques, will be instrumental to validate the conclusions, and achieve the full picture.

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Highlights

- Metal-based compounds form a promising class of therapeutic agents.
- Metallodrugs are pro-drugs which undergo activation processes and speciation in vivo.
- Molecular mass spectrometry is a major tool to study the interactions of metallodrugs at a molecular level.
- Non-covalent adducts of metal compounds with biomolecules can be detected by mass spectrometry.
- Mass spectrometry imaging of metallodrugs in complex samples provides invaluable information on their modes of action.
- New chemical rules have to be defined to characterize metallodrugs' interactions in biological systems.